OPTICALLY PURE D (-) LACTIC ACID BIOSYNTHESIS FROM DIVERSE RENEWABLE BIOMASS: MICROBIAL STRAIN DEVELOPMENT AND BIOPROCESS ANALYSIS

by

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
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Abstract

Lactic acid is an important platform chemical that has long history and wide applications in food, polymer, pharmaceutics and cosmetic industries. Lactic acid has two optical isomers; namely D-lactic acid and L-lactic acid. Racemic mixture of lactic acid are usually used as preservatives and ingredients in solvents, or as precursors for different chemicals. Currently there is an increasing demand of optical pure lactic acid as a feedstock for the production of poly-lactic acid (PLA). PLA is a biodegradable, biocompatible and environmental friendly alternative to plastics derived from petroleum based chemicals. Optically pure D or L-lactic acid is used for the synthesis of poly D or L- lactic acid (PDLA, PLLA). Blend of PDLA with PLLA results in a heat-resistant stereocomplex PLA with excellent properties. As a consequence, large quantity of cost effective D-lactic acid is required to meet the demand of stereocomplex PLA. Lignocellulosic biomass is a promising feedstock for lactic acid production because of its availability, sustainability and cost effectiveness compared to refined sugars and cereal grain-based sugars. Commercial use of lignocellulosic biomass for economic production of lactic acid requires microorganisms that are capable of using all sugars derived from lignocellulosic biomass. Therefore, the objectives of this study were: 1) to produce high level of optically pure D-lactic acid from lignocellulosic biomass-derived sugars using a homofermentative strain *L. delbrueckii* via simultaneous saccharification and fermentation (SSF); 2) to develop a co-culture fermentation system to produce lactic acid from both pentose and hexose sugars derived from lignocellulosic biomass; 3) to produce D-lactic acid by genetically engineered *L. plantarum* NCIMB 8826 Δ*ldhL1* and its derivatives; 4) to construct recombinant *L. plantarum* by introduction of a plasmid (pLEM415-*xylAB*) used for xylose assimilation and evaluate its ability to produce D-lactic acid from biomass sugars; and 5) to perform metabolic flux analysis of carbon flow in *Lactobacillus* strains used in our study.

Our results showed that D-lactic acid yield from alkali-treated corn stover by *L. delbrueckii* and *L. plantarum* NCIMB 8826 Δ*ldhL1* via SSF were 0.50 g g^{-1} and 0.53 g g^{-1} respectively; however, these two D-lactic acid producing strains cannot use xylose from hemicellulose. Complete sugar utilization was achieved by co-cultivation of *L. plantarum* ATCC 21028 and *L. brevis* ATCC 367, and lactic acid yield increased to 0.78 g g^{-1} from alkali-treated

corn stover, but this co-cultivation system produced racemic mixture of D and L lactic acid. Simultaneous utilization of hexose and pentose sugars derived from biomass was achieved by introduction of two plasmids pCU-P*xylAB* and pLEM415-*xylAB* carrying xylose assimilation genes into *L. plantarum* NCIMB 8826 Δ*ldhL1*, respectively; the resulting recombinant strains Δ*ldhL1*-pCU-P*xylAB* and Δ*ldhL1*-pLEM415-*xylAB* used xylose and glucose simultaneously and produced high yield of optically pure D-lactic acid. Metabolic flux analysis verified the pathways used in these *Lactobacillus* strains and provided critical information to judiciously select the desired *Lactobacillus* strain to produce lactic acid catering to the composition of raw material and the optical purity requirement. This innovative study demonstrated strategies for low-cost biotechnological production of tailor-made lactic acid from specific lignocellulosic biomass, and thereby provides a foundational manufacturing route for a flexible and sustainable biorefinery to cater to the fuel and chemical industry.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

CDS: condensed distillers solubles

CCR: carbon cartabolite repression

CcpA: carbon catabolite protein A

DDGS: dried distillers grains with solubles

EMP: Embden-Meyerhof pathway

FDA: food and drug administration

GAP: glyceraldehyde 3-phosphate

GRAS: generally recognized as safe

HMF: 5-hydroxylmethyfurfural

LAB: lactic acid bacteria

LDH: lactate dehydrogenase

PLA: poly-lactic acid

PLLA: poly-L-lactic acid

PDLA: poly-D-lactic acid

PK: phosphoketolase

SSF: simultaneous saccharification and fermentation

SHF: sequential hydrolysis and fermentation

SBME: soybean meal extract

YE: yeast extract

X5P: xylulose-5-phosphate

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Dedication

To my beloved parents and husband

Chapter 1 - Biosynthesis of lactic acid using *Lactobacilli strains*: an important platform chemical

Lactic Acid

Lactic acid (2-hydroxypropanoic acid) is an important and versatile chemical with a long history of use in the food and cosmetic Industries. Lactic acid was discovered by a Swedish chemist, Carl Wilhelm Scheele, in 1780. After a century, a French scientist produced lactic acid by bacterial fermentation and this lead to the industrial production of lactic acid (Ghaffar et al. 2014).

Lactic acid has two enantiomers, L-lactic acid and D-lactic acid due to its asymmetric C_2 atom (Fig.1.1). Both enantiomers have similar physical and chemical properties; the pK value of lactic acid is 3.89 and dissociates freely at pH around 7, yielding lactate (Ewaschuk et al. 2005). L-lactic acid is a biologically important isomer, which is a major cause of muscle fatigue and a key factor in acidosis-induced tissue damage (Gladden 2004). Table 1.1 summarizes the physical properties of these two enantiomers.

Applications of lactic acid

Lactic acid has many applications in the food, chemical, textile, pharmaceutical and other industries. Different fields prefer different isomer of lactic acid; in particular, the food and pharmaceutical industries have a preference for the L-lactic acid because it is the only one that can be metabolized by the human body. However, the chemical industry requires one of the pure isomers or a racemic mixture of both depending on the specific applications (Martinez et al. 2013).

Lactic acid is classified as GRAS (generally recognized as safe) by US Food and Drug Administration (FDA), and approximately 70 % of produced lactic acid is used in food industry (Martinez et al. 2013). Due to its acidic nature, lactic acid is often used as a natural flavoring in beverage, as an acidulant in confectionery, and as a preservative for fermented vegetables and meat. Lactic acid salt or esters with long chain fatty acid can be used as an emulsifying agent in bakery goods (Dusselier et al. 2013). Traditionally non-food related uses are found in the leather tanning and textile treatments as well as in pharmaceutical and cosmetic industries. Calcium

lactate trihydrate is used in pharmaceuticals as a dietary calcium source and also as a blood coagulant in the treatment of hemorrhages and to inhibit bleeding during dental operations. Sodium lactate is used in the production of some antibiotics and buffering systems (Vijayakumar et al. 2008). Natural L-lactic acid is used as a skin-rejuvenating agent and pH regulator in the cosmetic industry; for example, the use of ethyl lactate in anti-acne treatments, and sodium lactate as moisturizing agents in many skin care products. (Datta and Henry 2006).

Lactic acid has a hydroxyl and a carboxyl functional group; it can undergo intermolecular or self-esterification and form poly-lactic acid (PLA), which is well-known as a sustainable bio-plastic (Datta et al. 1995). PLA has numerous applications such as industrial packaging, fibers, clothes, and biocompatible materials for medical application. (Jamshidian et al. 2010). The optical purity of lactic acid plays important role in PLA physical properties; it can be semi-crystalline or totally amorphous depending on the stereo-purity of the polymer backbone. The polymers derived from optically pure D or L-lactic acid are semi-crystalline materials, while the polymer derived from racemic DL-lactic acid is usually amorphous (Garlotta 2001). The amorphous PLA is usually used for drug delivery, because it is important to homogeneously disperse the active ingredients in the carrier matrix; on the other hand, the semi-crystalline PLA is preferred in applications where high mechanical strength and toughness are required (Tsuji and Ikada 1999a). Blending of poly-L-lactic acid (PLLA) with poly-D-lactic acid (PDLA) results in stereocomplex PLA which has superior heat-resistant property than respective single polymers (Okano et al. 2007). Table 1.2 shows the physical properties of PLA.

Lactic acid industry

Currently the major lactic acid producers in the United States are Nature Works LLC and Archer Daniels Midland Company (ADM). In Europe, there are Purac (The Netherlands) and Galactic S. A (Belgium); and in Asia, Musashino Chemical Co. Ltd (Japan), Changzhou (CCA) Biochemical Co. Ltd., and Henan Jindan Lactic Acid Co. LTD (China). ADM mainly focuses on lactic acid and its derivatives for traditional uses, while Nature works LLC has been the dominant leader in PLA business. Musashino Chemical Co. Ltd manufactures lactic acid by carbohydrate fermentation technology with several Chinese partners (Datta and Henry 2006) .

According to a business report published by Grand View Research, Inc (“Lactic acid”, 2014) The current worldwide production (including polymer uses) is estimated to reach around

1960 kilo tons by 2020, with an annual growth rate of 15.5 % from 2014 to 2020. Industrial applications accounted for 44.3 % of the overall market in 2013, and are expected to continue dominating the market over the forecast period. Major driving forces of lactic acid market are PLA and lactate solvents as well as personal care products. The demand for sustainable packaging products as well as the rising crude oil prices is expected to drive demand for the PLA. Although the demand for PLA is increasing, its current production capacity is only 496 kilo tons per year (Ghaffar et al. 2014), which results from the high manufacturing cost of its monomer-lactic acid. The selling price of PLA must decrease by about half of its current selling price in order to compete with its petroleum counterparts, which means that lactic acid price should be at or below $0.8 per kilogram (Okano et al. 2010).

Lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) are the most widely used bacterial group for industrial production of lactic acid, because of their long history in industrial scale production and they are safe to the consumers and production workers (Vijayakumar et al. 2008). LAB are gram-positive microorganisms widely present within plants, meat, and dairy products, they produce lactic acid as an anaerobic product of glycolysis with high yield and productivity. Most of the LAB are anaerobic, but some of them, for example *Lactobacillus* species are facultative anaerobes; they can also grow in presence of oxygen due to the presence of peroxidases (Ghaffar et al. 2014). LAB are generally divided into three groups according to their fermentation patterns. The first group is homofermentative LAB, which produce lactic acid as the major end product. Theoretical maximal yield of lactic acid is 2 moles lactic acid per mole of glucose or 1 gram lactic acid per gram of glucose. Representative LAB in this group are species of *Lactococci*, *Enterococci*, and *Streptococci* (Taskila and Ojamo 2013). Homo-lactic acid bacteria metabolize hexose to pyruvate through the Embden-Meyerhof pathway (EMP). Then the pyruvate is used to regenerate NAD^+ in the lactate dehydrogenase (LDH) catalyzed reaction yielding lactic acid. There are two types of LDH, namely L-LDH and D-LDH, which are responsible for catalyzing reactions for L-lactic acid or D-lactic acid, respectively. Some species have both L-LDH and D-LDH; thus a racemic mixture of D/L lactic acid is produced. Heterofermentative LAB, for example *Leuconostocs*, *Oenococci* and some *Lactobacilli*, for example, *Lactobacillus brevis*, which can use both hexose and pentose sugars *via* phosphoketolase (PK) pathway (Zaunmueller

et al. 2006). The theoretical maximal yield of lactic acid in hetero-LAB is 1 mole of lactic acid per mole of glucose or 0.5 gram of lactic acid per gram of glucose. They also yield 1 mole of lactic acid per mole of xylose or 0.6 gram of lactic acid per gram of xylose (Tanaka et al. 2002). Hetero-LAB also produce by-products such as acetic acid, ethanol or some polyols. Besides homo-and heterofermentative LAB, a third group of LAB known as the facultative heterofermentative LAB, metabolize hexose through EMP, but they also possess an inducible PK pathway with pentose acting as an inducer (Kandler 1983). Different pathways are described in Fig.1.2.

Commercially important LAB strains, such as *Lactobacillus* strains, are particularly useful because of their high lactic acid yield, high acid tolerance and their ability to be metabolically engineered. Several studies have been done to improve lactic acid production by gene modification of LAB. Usually, LAB are modified in two aspects; one is to improve the optical purity of lactic acid and minimize the by-product production. The other strategy is to expand the substrate utilization profile of LAB. D-lactic acid has attracted intensive attention due to the rapid growth of PLA industry. Gene manipulation of D-lactic acid producing strains is known to be difficult due to the lack of effective transformation method and compatible plasmid (Serror et al. 2002). Therefore, considerable research has been done in the manipulation of the D,L-lactic acid producing strains. Okano et al. (2009a; 2009b) has deleted the L-LDH gene of *L. plantarum* NCIMB 8826, and xylose assimilating genes were introduced into the mutant strain resulting in successful production of D-lactic acid from glucose, arabinose and xylose with optical purity above 99 %. Direct lactic acid production from starch was achieved by cloning and expressing a-amylase in *L. lactis* IL 1403, the resulting strain produced L-lactic acid from soluble starch directly (Okano et al. 2009c). Direct fermentation from cellulose to produce lactic acid is a challenge. So far, there is no report about direct lactic acid production from cellulosic biomass. Though direct lactic acid production was achieved from cello-oligosaccharides consisting of more than 4 glucose units (Adsul et al. 2007).

Lactic acid production from lignocellulosic biomass

Lactic acid can be produced by either chemical synthesis or fermentation. The chemical synthesis route produces a racemic mixture of D, L lactic acid (Dusselier et al. 2013). Due to the growing demand of lactic acid for biodegradable PLA, the need for optically pure lactic acid is

increasing. On the other hand, the fermentation route mainly produces optically pure lactic acid if an appropriate microorganism is selected. Commercial lactic acid is produced by fermentation of starch or refined sugars. The expensive raw materials account for a large portion of production cost, representing the most serious obstacle for fermentative lactic acid while competing with synthetic lactic acid production (Datta et al. 1995). Lactic acid is a commodity chemical required by other industrial users in large quantities with a relatively low cost. Therefore, low-cost raw materials are necessary for the feasibility of the biotechnological production of lactic acid. Low cost, renewable, and non-food materials are of great interest for lactic acid production, especially lignocellulosic biomass from agricultural, agro-industrial, and forestry sources (Yadav et al. 2011). However, the cellulose and hemicellulose in lignocellulosic biomass cannot be directly used by LAB to produce lactic acid because of the complex structure of lignocellulosic biomass and the lack of cellulolytic enzymes in LAB.

Lignocellulosic biomass composition

Lignocellulosic biomass is organic material, which is generally categorized into woody biomass, agricultural residues including straws, bagasse and stover, and organic waste. Lignocellulosic biomass is mainly composed of cellulose, hemicellulose and lignin. Cellulose is a linear polysaccharide polymer with many glucose unit linked with β-1, 4 glycosidic bond. It is the major component in the rigid cell walls in plants. Because of the orientation of the linkages and additional hydrogen bond formed on the same or on neighbor chains, cellulose is highly crystalline and hard to break (Abdel-Rahman et al. 2011). Unlike cellulose, hemicellulose is highly branched heteropolymer consisting of xylan, glucuronoxylan, arabinoxylan, glucomannan, and xyloglucan (Saha 2003). Hemicellulose is much easier to hydrolyze compared with cellulose due to its amorphous structure. Xylose is the dominant sugar from hemicellulose in hardwoods and agriculture residues, while mannose is the major hemicellulose sugar in soft woods (Taherzadeh and Karimi 2008). Lignin is a complex aromatic polymer, composed of up to three monomers (coumaryl alcohol, coniferyl alcohol and sinapyl alcohol), and a complex matrix is formed by many possible bonding patterns between individual units (Demirbas 2008). Lignin is considered to be difficult to use as a fermentation substrate and is usually removed during pretreatment. The composition of biomass varies between species. Table 1.3 shows the composition of difference source of biomass. It is clearly evident that the cellulose,

hemicellulose and lignin content depends on the type of biomass; therefore, by selecting appropriate biomass and LAB, the fermentation purpose can be tailor-made for a defined application.

Pretreatment methods

The biologic process of converting lignocellulosic biomass to lactic acid usually consists of three major steps: pretreatment, enzymatic hydrolysis and fermentation (Fig. 1.4). The purpose of pretreatment is to disrupt lignocellulosic biomass structure to make cellulose more accessible to the enzymes. The removal of lignin and hemicellulose during pretreatment depend on the methods used. For example, dilute acid pretreatment, which is a leading pretreatment process under commercial development, removes most of the hemicellulose from biomass (Xu and Huang 2014).

In dilute acid pretreatment, the lignocellulosic biomass is treated with less than 4 % sulfuric acid at a temperature of 120 to 210℃ for several minutes to an hour (Taherzadeh and Karimi 2007). Dilute acid pretreatment is effective to improve cellulose and hemicellulose hydrolysis by varying its severity. The disadvantage of this treatment is the formation of inhibitors such as furfurals and 5-hydroxymethyfurfural (HMF) for the subsequent hydrolysis and fermentation steps. Alkaline pretreatment, for example, sodium hydroxide pretreatment and lime pretreatment are usually performed under lower temperature and pressure compared to dilute acid pretreatment. Alkaline pretreatment is a delignification process, which removes most lignin from the biomass, and the swollen cell wall increases the internal surface area, which makes the cellulose more accessible for enzymes (Xu et al. 2010). Alkaline pretreatment forms less amount of furfurals and HMF compared to diluted acid pretreatment. (Taherzadeh and Karimi 2008). Liquid hot water pretreatment is a neutral pretreatment, which is usually performed in bath or flow-through reactors. The slurry of biomass and water is usually heated to 160 to 240 ℃ for a few minutes to an hour (Sanchez and Cardona 2008). The major advantage of liquid hot water pretreatment is that no acid catalysts are required, which minimizes the formation of inhibitors and also avoids the final washing step or neutralization step. The disadvantage of this method is high energy demand because of high pressure and large amount of water used. Ozonolysis is carried out at temperature of 20-30 ℃, and ozone is supplied at flow rate from 0.5 to 0.8 L/min (Taherzadeh and Karimi 2008). Unlike other pretreatments,

Ozonolysis does not produce toxic compounds, which is a major advantage of this method, but large amount of ozone is needed, which makes the process expensive and less applicable (Garcia-Cubero et al. 2009).

Enzymatic hydrolysis

The goal of enzymatic hydrolysis is to depolymerize cellulose and hemicellulose remaining after pretreatment step into fermentable sugars. To maximize sugar yield, a mixture of enzymes are needed. There are two general categories of enzymes: cellulase and hemicellulase. Synergistic reaction of three major groups of cellulases (endo-β-1, 4-glucanases, exo- β-1, 4-glucanases and β-glucosidases) are required for efficient cellulose degradation (Zhang et al. 2007). Endo- β-1, 4-glucanases randomly cut intramolecular β-1, 4-glycosidic bonds of cellulose chains. Exo- β-1, 4-glucanases and β-glucosidases hydrolyze cellulose chains at the ends of the polymer to produce soluble cellobiose and glucose. β-glucosidases cleave cellobiose into two glucose (Lynd et al. 2002). Enzymatic hydrolysis of hemicellulose requires endo-1, 4- β-xylanase, β-xylosidase, β-glucuronidase, α-L-arabinofuranosidase and acetylxylan esterase for hydrolyzing xylan (Carvalheiro et al. 2008). β -mannanase and α-mannosidase are required to cleave glucomannan (Kumar et al. 2008).

Fermentation process with LAB

The lignocellulosic biomass hydrolysate is a mixture of glucose, xylose, arabinose and/or mannose depending on the type of biomass. Glucose can be easily metabolized by LAB to form lactic acid, however, most LAB lack the enzymes for metabolizing xylose. Hinman et al (1989) stated that the complete utilization of biomass-derived sugars can reduce the manufacturing cost of biomaterials by as much as 25 %. Table 1.4 shows lactic acid production from different lignocellulosic biomass by LAB. Fermentation technologies must be cost competitive when compared to chemical synthesis of lactic acid to validate the use of biotechnologies on industrial scale (Bustos et al. 2007). Most studies focus on improving product yield, productivity and product concentration of lactic acid, which are the three main economic drivers in fermentation process (Taskila and Ojamo 2013).

Effect of pH

Most LAB cannot grow under the pH of 4 (Adachi et al. 1998). Low pH of lactic acid has an inhibitory effect on cellular metabolism and lactic acid production, therefore neutralizing agent need to be added such as sodium hydroxide, calcium carbonate, ammonium hydroxide through the fermentation process to keep the pH constant in order to reduce the inhibitory effect of low pH. The desired pH for lactic acid production by majority of LAB was found to be 5-7 (Hofvendahl and Hahn-Hagerdal 2000). pH controlled batch fermentation significantly increased lactic acid yield and productivity compared to pH uncontrolled batch fermentation by different LAB strains, such as *L. delbrueckii* (Tashiro et al. 2011). Nakano et al. (2012) reported that Ca^{2+} is better than monovalent cation (Na^{+}, NH_4^{+}) as neutralizing agent for lactic acid production in simultaneous saccharification and fermentation by *L. delbrueckii* JCM1106.

Effect of temperature

Temperature is one of the important factors that affects lactic acid production. The majority of LAB, such as, *L. delbrueckii*, are mesophilic bacteria, which grow at 17-50℃ and have optimum growth temperature between 37-43℃ (Hofvendahl and Hahn-Hagerdal 2000). Goksungur and Guvenc (1997) reported the optimal temperature for *L. delburueckii* IFO 3202 is at 45℃; when the temperature increased above 45℃, lactic acid production and yield decreased rapidly.

Simultaneously saccharification and fermentation (SSF)

SSF is a good strategy for lactic acid production from lignocellulosic biomass; it combines enzymatic hydrolysis and fermentation into a single step and has many advantages compared to separate hydrolysis and fermentation (SHF). SSF reduces reactor volume, processing time and feedback inhibition, and consequently increases the productivity, lactic acid yield and hydrolysis rate, and it also reduces the enzyme loading (Hofvendahl and Hahn-Hagerdal 2000).

Enzymes involved in the hydrolysis step are known to be subjected to feedback inhibition by the sugars released from lignocellulosic biomass (Olofsson et al. 2008). One advantage of SSF is that the microorganisms can quickly consume the sugars and maintain the concentration of sugar at a low level, thus significantly reducing the feedback inhibition (Balat et al. 2008). In SSF, hydrolysis is usually the rate-limiting step (Philippidis and Smith 1995). Production of lactic acid by SSF has been studied using corn stover, corncob, waste wood, wheat straw and

alfalfa fiber (Sreenath et al. 2001; Garde et al. 2002; Lee et al. 2004; Miura et al. 2004; Cui et al. 2011). The disadvantage of SSF is that the difference of optimum temperature and pH required for saccharification and fermentation (Huang et al. 2005), and lactic acid inhibition effect on the enzymes; however, the lactic acid inhibition factor is much lower than the feedback inhibition caused by sugar buildup (Takagi 1984).

Mixed culture fermentation

Mixed cultures have been used in the dairy industry for cheese and yogurt production. In the mixed culture system, LAB demonstrated a symbiotic relationship (Galeslcoo et al. 1968). Mixed culture have also been used in lactic acid production to increase the conversation efficiency of substrates as well as lactic acid yield (Nancib et al. 2009, Cui et al. 2011).

There is more than one sugar present in lignocellulosic hydrolysate, and it is difficult to separate them, thus microorganisms are required to produce lactic acid from both glucose and xylose. A co-cultivation system which involve microorganisms that are suitable for each sugar present will be useful. Taniguchi et al. (2004) reported a co-culture system with a two stage inoculation using a xylose consuming strain *E. casseliflavus* and a glucose specific strain *Lactobacillus casei* to produce lactic acid from a mixture of glucose and xylose. *L. casei* was inoculated first to consume glucose in the medium followed by *E. casseliflavus* inoculation due to the carbon catabolite repression. Lactic acid (95 g L^{-1}) with high optical purity was obtained by 192 h with all the sugars consumed. Cui et al. (2011) cultivated *Lactobacillus rhamnosus* along with *Lactobacillus* brevis. *L. rhamnosus* consumed glucose rapidly to give lactic acid at high productivity; *L. brevis* simultaneously converted both glucose and xylose to lactic acid with a small amount of acetic acid. Lactic acid yield and productivity increased when these two strains were co-cultivated. All these results suggest that the mixed cultures of LAB are efficient and could be more efficient than single cultures regarding lactic acid concentration and better sugar utilization. The existence of possible synergistic interaction among LAB should be studied in more depth.

Challenges of using lignocellulosic biomass for lactic acid production

Using lignocellulosic biomass to produce chemicals is a promising way to possibly solve the energy crisis problem; however, efficient conversion of biomass to lactic acid still faces considerable challenges. One of them is the difficult biomass hydrolysis for efficient sugar

utilization by microorganisms (Kumar et al. 2008). A large number of pretreatment methods have been studied, but the high energy demand for pretreatment is a major drawback and affects the total economy of the bioconversion of lignocellulosic biomass (Zheng et al. 2009). Moreover, inhibitory compounds usually are released during the pretreatment process; these inhibitory compounds interfere with cellulase hydrolysis of lignocellulosic biomass and some of them have been identified as microbial growth inhibitors (Mussatto and Roberto 2004). Also the high costs of enzymes and excessive dosage of enzymes are some of the drawbacks that limit commercial application of lignocellulosic biomass in the lactic acid industry (Abdel-Rahman et al. 2011). Another obstacle is that currently only hexoses derived from lignocellulosic biomass can be easily used by LAB, while pentose sugars especially xylose cannot be fermented by most LAB (Tanaka et al. 2002). Only a few LAB metabolize pentose sugars through phosphoketolase pathway, which generate equimolar of lactic acid and acetic acid, and the yield of lactic acid from xylose is low. This co-production of lactic acid and acetic acid also increases the downstream purification cost. To achieve maximum product yield and productivity, the complete utilization of all sugars derived from lignocellulosic biomass is essential. Sequential sugar utilization is commonly found in many LAB, and this sequential utilization of mixed sugars make the fermentation process less effective (Bothast et al. 1999). A few LAB strains have been reported to simultaneously consume glucose and xylose; for example, *L. brevis* (Kim et al. 2009; Guo et al. 2010). Also mixed culture fermentation has been applied to maximize yield and productivity (Cui et al. 2011; Taniguchi et al. 2004). Isolation of superior LAB strains that produce less acetic acid or development of genetically engineered LAB strains are needed for efficient lignocelluloses utilization.

Table 1.1 physical properties of lactic acid

	Reported value	Enantiomer
Melting point ℃	52.7-53.0	L or D
	16.4-18.0	Racemic
Boiling point ℃ (at 1.87 kPa)	103	L or D
	122	Racemic
Solid Density g mL^{-1} (at 20 ℃)	1.33	–
Dissociation constant pKa	3.79-3.86	L or D
	3.73	racemic

*adapted from Dusselier et al. (2013)

Table 1.2 Physical properties of PLA

PLA structure	Description	Melting point T_m ℃	Glass transition point T_g ℃	Reference
PLLA or PDLA	LLLLLLLL or DDDDDD	170-190	55-65	Tsuji and Ikada 1996
Random optical copolymers	Random level of meso or D-lactide in L-lactide For example: LLDLLLDDLDLL	130-170	45-65	Baratian et al. 2001
PLLA/PDLA stereocomplex	LLLLLLLLL mixed with DDDDDD	220-230	65-72	Tsuji and Ikada 1999b
Poly meso lactic acid	DLDLDLDLDLDLDL	152	40	Ovitt and Coates 2002

Table 1.3 Cellulose, hemicellulose and lignin content of different type of lignocellulosic biomass (% dry weight)

Lignocellulosic biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Corn stover	36.8	30.6	23.1
Corn cobs	33.7-41.2	31.9-36	6.1-15.9
Corn stalks	35-39.6	16.8-35	7-18.4
Wheat straw	32.9-50	24-35.5	8.9-17.3
Softwood barks	18-38	15-33	30-60
Hardwood barks	22-40	20-38	30-55
Poplar aspen	50.8-53.3	26.2-28.7	15.5-16.3
Paper	85-99	0	0-15
Chemical pulps	60-80	20-30	2-10

*adapted from Xu and Huang (2014), Balat (2011)

Table 1.4 Lactic acid production from lignocellulosic biomass by *Lactobacilli*

LAB	Substrate	Fermentation mode	Lactic acid ($g\ L^{-1}$)	Yield ($g\ g^{-1}$)	Productivity ($g\ h^{-1}\ L^{-1}$)	Type	Reference
L. brevis S3F4	Corn stover hydrolysate	Batch	18.2	0.74	0.76	−	Guo et al. 2010
L. brevis CHCC 2097 and *L. pentosus* CHCC 2355	Wheat straw hydrolysate	Batch	8.0	0.74	0.08	D/L	Garde et al. 2002
L. brevis ATCC 367 and *L. rhamnosus*	Corn stover	SSF	21	0.7	0.58	D/L	Cui et al. 2011
L. delbrueckii ATCC 9649	Corn stover	SSF	20.1	0.50	0.32	D	Zhang and Vadlani 2013
L. delbrueckii ATCC 9649	Pulp	SSF	19.2	0.48	0.31	D	Zhang and Vadlani 2013
L. brevis ATCC 367 and *L. plantarum* ATCC 21028	Corn stover	SSF	31.2	0.78	0.43	D/L	Zhang and Vadlani 2015
L. brevis ATCC 367 and *L. plantarum* ATCC 21028	Poplar hydrolysate	Batch	38.0	0.80	0.40	D/L	Zhang and Vadlani 2015
L. pentosus ATCC 8041	Corn stover	Fed-batch (SSF)	74.8	0.65	0.05	−	Zhu et al. 2007
L. plantarum Δ*ldhL1*-pCU-P*xylAB*	Sorghum stalks	SSF	21.6	0.54	0.64	D	Chapter-5
L. plantarum Δ*ldhL1*-pLEM415-*xylAB*	Corn stover	Fed-batch (SSF)	61.4	0.77	0.32	D	Chapter-6

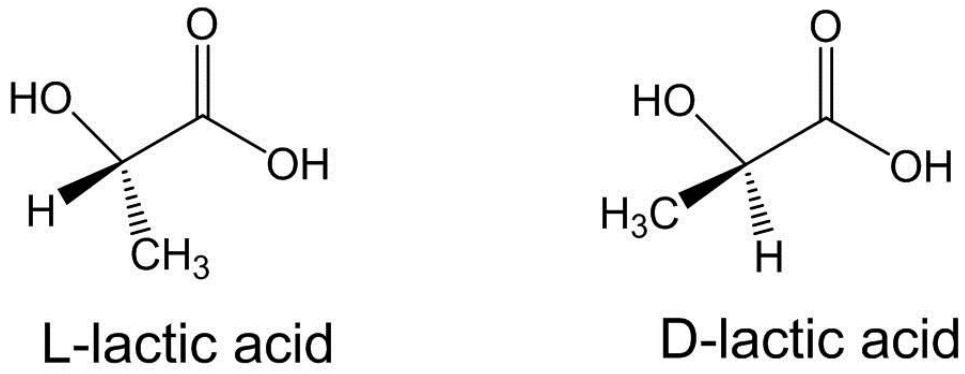

Figure 1.1 Lactic acid isomers

Source: http://nptel.ac.in/courses/116102006/module6/chapter%206.1.html

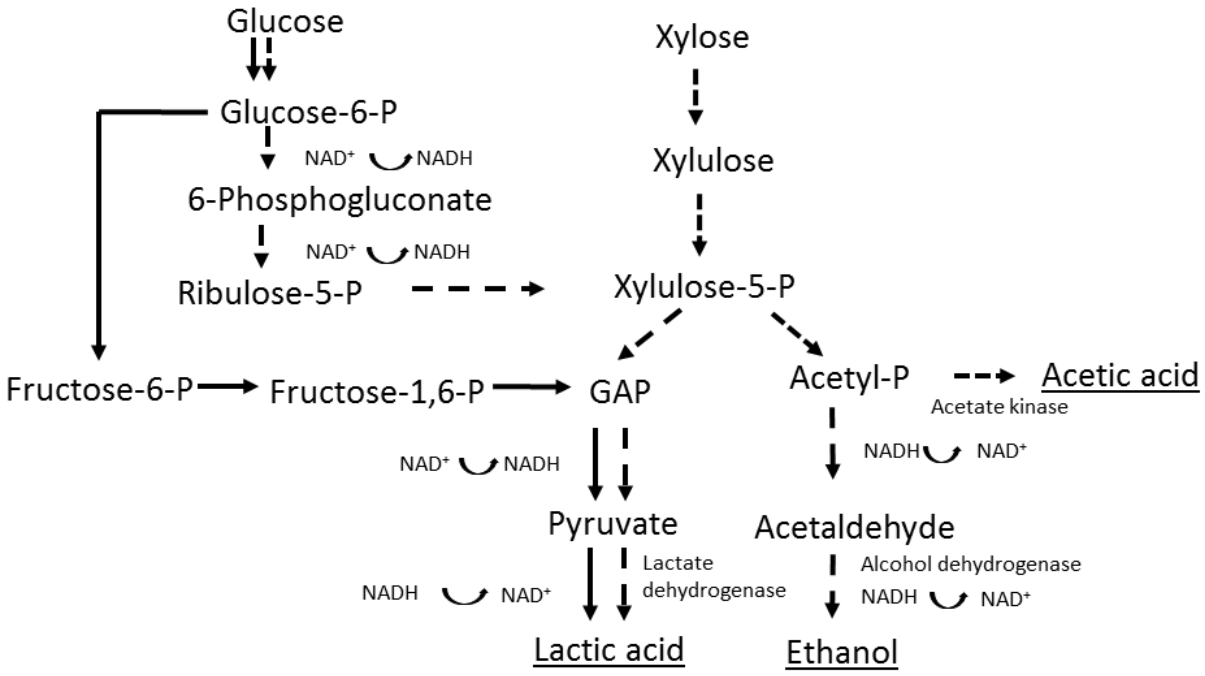

Figure 1.2 Simplified pathways in LAB

Homofermentative pathway (solid line), heteroformentative pathway (dashed line)

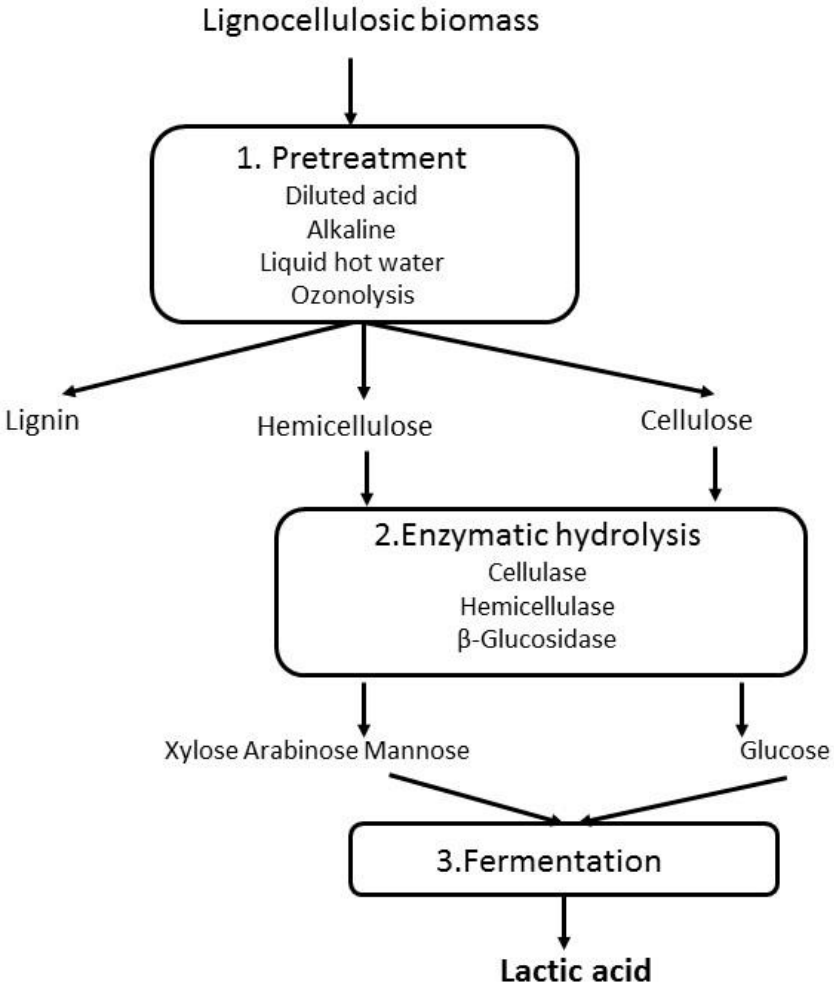

Figure 1.3 Schematic flow sheet of converting lignocellulosic biomass into lactic acid

Chapter 2 - Research objectives

Lactic acid has attracted intensive attention as an important platform chemical. The increased demand for biodegradable poly-lactic acid (PLA) and green solvents, e.g. ethyl lactate, has significantly increased the global interests in lactic acid production. However, the application of PLA is hindered by its high manufacturing cost due to the high price of optically pure D-lactic acid, which is not commercially available in large quantity. Therefore, the goal of this study was to economically produce high optically pure D-lactic acid through utilization of inexpensive lignocellulosic biomass, and to promote lactic acid yield and productivity by broadening the substrate specificity of lactic acid bacteria as well as applying advanced bioprocess techniques. Specific objectives corresponding to each chapter in this dissertation are listed below:

- Produce high level of optically pure D-lactic acid from lignocellulosic biomass-derived sugars using a homofermentative strain *L. delbrueckii via* simultaneous saccharification and fermentation. (Chapter-3)
- Develop a co-culture fermentation system to produce lactic acid from both pentose and hexose sugars derived from lignocellulosic biomass. (Chapter-4)
- Produce D-lactic acid by genetically engineered *L. plantarum* NCIMB 8826 Δ*ldhL1* and its derivatives. (Chapter-5)
- Construct recombinant *L. plantarum* by introduction of a plasmid (pLEM415-*xylAB*) used for xylose assimilation and evaluate its ability to produce D-lactic acid from biomass sugars. (Chapter-6)
- Perform metabolic flux analysis of carbon flow in *Lactobacillus* strains used in our study. (Chapter-7)

Chapter 3 - D-lactic acid biosynthesis from biomass-derived sugars via *Lactobacillus delbrueckii* fermentation[1]

Abstract

Poly-lactic acid (PLA) derived from renewable resources is considered to be a good substitute for petroleum-based plastics. The number of poly L-lactic acid applications is increased by the introduction of a stereocomplex PLA, which consists of both poly-L and D-lactic acid and has a higher melting temperature. To date, several studies have explored the production of L-lactic acid, but information on biosynthesis of D-lactic acid is limited. Pulp and corn stover are abundant, renewable lignocellulosic materials that can be hydrolyzed to sugars and used in biosynthesis of D-lactic acid. In our study, saccharification of pulp and corn stover was done by cellulase CTec2 and sugars generated from hydrolysis were converted to D-lactic acid by a homofermentative strain, *L. delbrueckii*, through a sequential hydrolysis and fermentation process (SHF) and a simultaneous saccharification and fermentation process (SSF). D-lactic acid (36.3 g L^{-1}) with 99.8 % optical purity was obtained in the batch fermentation of pulp and attained highest yield of 0.83 g g^{-1} and productivity of 1.01 g $L^{-1}h^{-1}$. Luedeking-Piret model described the mixed growth-associated production of D-lactic acid. A maximum specific growth rate of 0.2 h^{-1} and product formation rate of 0.026 h^{-1}, were obtained for this strain. The efficient synthesis of D-lactic acid having high optical purity and melting point will lead to unique stereo-complex PLA with innovative applications in polymer industry.

Introduction

Lignocellulosic biomass is gaining importance as a potential source of renewable energy and chemicals as the fossil fuel reserves will eventually getting depleted. Demand continues to increase for production of high-value chemicals and materials from renewable resources to attain domestic self-sufficiency and enhanced national security. Lactic acid is an important and multifunctional organic acid that has wide applications in the food, pharmaceutical, and chemical industries (Shen and Xia 2006; Datta et al.1995) It exists in two optical isomeric forms, L (+)

[1] Chapter 3 is published as a part of Zhang Y and Vadlani PV (2013) Bioprocess and Biosystems Engineering 36:1897-1904

and D (-) poly-lactic acid (PLA), which are being developed as a substitute for petroleum-derived plastics. The high chemical resistance of poly lactic acid is advantageous in the manufacture of fibers, nonwoven fabrics, and films (Tanaka et al. 2006); however, the application of poly L-lactic acid (PLLA) is limited by its melting point (Brizzolara et al. 1996). This problem can be obviated by blending it with poly D-lactic acid (PDLA). The melting point of the resulting stereocomplex polymer is approximately 50 °C higher than that of the respective single polymers (Ikada et al. 1987). The optical purity of lactic acid accentuates the physical properties of poly D-lactic acid-based polymers (Tsuji 2002). The chemical process of making lactic acids usually yields a mixture of these two enantiomers, which is an undesirable feature; therefore, the biological process of making pure lactic acid is preferred (Yadav et al. 2011).

To date, intense studies have been conducted on the production of L-lactic acid from different biomass through microorganism microbial fermentation (Vadlani et al. 2008a; 2008b; Moon et al. 2012a; Phrueksawan et al. 2012), but information on biosynthesis of D-lactic acid from biomass is limited. A few wild-type strains such as *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Sporolactobacillus inulinus* (Fukushima et al. 2004), *Lactobacillus coryniformis* subsp. *torquens* (Yanez et al. 2003), and *Lactobacillus delbrueckii* subsp. *lactis* QU41 (Tashiro et al. 2011) have been identified as D-lactic acid producers. Traditional production of lactic acids typically uses starch derived from food crops as the fermentation substrate (Fukushima et al. 2004; Shinkawa et al. 2009), but this process may affect the global food supply. Lignocellulosic materials are favorably structured to produce lactic acids, which require the breakdown of cellulose to sugars (Schmidt et al. 1997). This step usually can be done by acid hydrolysis and enzymatic hydrolysis. The enzymatic hydrolysis method is preferred, because it can be done under mild reaction conditions and it avoids using avoiding the use of toxic and corrosive chemicals (Xu et al. 2007). The hydrolysis and fermentation steps can be done sequentially (SHF) or simultaneously (SSF). The SSF process offers better yields because it avoids product inhibition and results in higher productivity (Marques et al. 2008; Kim et al. 2003).

Production of D-lactic acid from cardboard (Yanez et al. 2005a; 2005b), cellulose (Yanez et al. 2003), peanut meal (Wang et al. 2011), and rice bran (Tanaka et al. 2006) has been studied. Other sources include pulp and corn stover, which have the potential to become cheap and abundant sources for production of ethanol, organic acids, and other chemicals (Yadav et al. 2011; Yanez et al. 2005b). Pulp is prepared by chemically or mechanically separating cellulose

fibers from wood, fiber crops, or waste paper (Biermann 1996). Corn stover, which includes the leaves, stalks, and cobs of corn plant, is the most abundant agricultural residue in the U.S. (Li et al. 2004); to the best of our knowledge, no research has been reported on D-lactic acid fermentation via pulp and corn stover as substrates.

The purpose of this study was to produce D-lactic acid with high yield and optical purity from pulp and corn stover by *Lactobacillus delbrueckii* ATCC 9649. *L. delbrueckii* is a homofermentative lactic acid bacterium that can provide a continuous bioprocess with high volumetric productivity and high optically purity of D-lactic acid under anaerobic conditions (Calabia and Tokiwa 2007). In addition, kinetic analyses of enzyme hydrolysis and fermentation of glucose to D- lactic acid also have been studied in this work.

Materials and methods

Raw materials and chemical treatment

Regular pulp and mechanically modified pulp were obtained from the MeadWestvaco's Crompton mill. Corn stover was obtained from fields in Manhattan and Tribune, Kansas. Alkali treatment was performed on corn stover before hydrolysis. Corn stover was suspended in 20 g L^{-1} NaOH and heated at 121 °C for 30 min in an autoclave (Tomy SS-325E, Tomy SEKO CO., LTD, Tokyo, Japan), then washed under running distilled water and filtered through muslin cloth until no color was visible in the wash water. The alkali-treated corn stover was dried at 80 °C for 24 h and ground to fine particle size in a laboratory mill (3303, Perten Instruments, Springfield, IL) for further enzymatic hydrolysis.

Enzyme hydrolysis

CTec2 (cellulase) obtained from Novozymes Inc. (Franklinton, NC) was used in this experiment. Enzyme hydrolysis assays were carried out at 45 °C in 250 mL screw capped plastic conical flasks with orbital agitation (150 rpm). The substrate concentration was 2 % (w/v). pH was kept at 4.8 using 0.05 mol L^{-1}citric acid-sodium citrate buffer. The cellulase activity of CTec2 was measured by the filter paper assay (Ghose 1987), and the activity was expressed in terms of filter paper units (FPU). CTec2 was added on a dosage of 2, 4, and 8 FPU g^{-1} of dry biomass, respectively. Product yield is based on the amount of glucose released divided by the amount of biomass consumed.

Microorganism and culture conditions

Lactobacillus delbrueckii ATCC 9649 obtained from the American Type Culture Collection (Manassas, VA) was used in this work. *L. delbrueckii* inoculum was prepared by growing cells in a 100 mL Wheaton serum bottle containing 50 mL of liquid MRS medium (MRS broth, Difco Laboratories, Detroit, MI) and incubated at 37 °C in a temperature-controlled shaker (Innova 4300, New Brunswick scientific, NJ) at 120 rpm for 15 h. CO_2 (3 vvm) was sparged into the bottle to create anaerobic growing conditions.

Sequential hydrolysis and fermentation (SHF)

Shake flask fermentation was modified according to the procedure described by Mukhopadhyay (2009). Fermentation was performed in 100 mL Wheaton serum bottles containing 50 mL of synthetic medium, pulp, modified pulp, or corn stover hydrolysate, and lasted for 30 h. The synthetic medium consisted of 10 g L^{-1} of glucose, 10 g L^{-1} of peptone, 5 g L^{-1} of yeast extract, 2 g L^{-1} of ammonium citrate, 2 g L^{-1} of sodium acetate, 2 g L^{-1} of K_2HPO_4, 0.1 g L^{-1} of $MgSO_4.7\ H_2O$, 0.05 g L^{-1} of $MnSO_4.4H_2O$, and 1 g L^{-1} of Tween 80. Pulp, modified pulp, and corn stover hydrolysate were supplemented with all the components (except glucose) of the synthetic medium. pH of the medium was adjusted to 6.5 by 10 mol L^{-1} NaOH, and 3% (w/v) of calcium carbonate was added to control the pH. Temperature was maintained at 37 °C, and agitation was 120 rpm.

Batch and fed-batch fermentation were performed in a 7 L fermenter with a working volume of 5 L (Bioflo 110, New Brunswick Scientific Inc. Enfield, CT). In the batch fermentation experiment, paper pulp was added in quantity (270 g) that would possibly achieve a glucose concentration of 40 g L^{-1} in the medium. After hydrolysis, the pulp hydrolysate was supplemented with all the components (except glucose) of the synthetic medium. The synthetic medium was used in fed-batch fermentation as a control. After 36 h, 1 L of fermentation medium was taken out and 1 L of feeding medium, which consisted of 40 g L^{-1} of glucose, 2 g L^{-1} of ammonium citrate, 2 g L^{-1} of sodium acetate, 2 g L^{-1} of ammonium citrate, 2 g L^{-1} of K_2HPO_4, 0.1 g L^{-1} of $MgSO_4.7\ H_2O$, and 0.05 g L^{-1} of $MnSO_4.4H_2O$, was added. During the fermentation, the temperature was maintained at 37 ºC; agitation speed at 100 rpm; and pH at 6.5. CO_2 was sparged at 3 vvm through the vessel to maintain anaerobic conditions.

Simultaneous saccharificaiton and fermentation (SSF)

SSF process was modified according to the procedure described by Mukhopadhyay (2009) performed in 100 mL Wheaton serum bottles. The optimal temperature and pH for the enzyme and the bacterium are different, In SSF, temperature was set up at 40 °C and pH was at 5.5, which was considered a suitable condition for both enzyme and bacterium. Two gram of dried pulp and corn stover was suspended in 50 mL 0.05 mol L^{-1} sodium citrate buffer (pH 5.5) with all the components (except glucose) of the synthetic medium. 3 % (w/v) calcium carbonate was added to control the pH. CTec2 was added at 8 FPU g^{-1} of biomass, and *L. delbrueckii* was inoculated at 5 % (v/v). Incubation temperature was 40 °C, and agitation rate was 150 rpm.

Analyses

Fermentation samples were centrifuged at 15,000×g for 10 min in an Eppendorf centrifuge (5415R, Eppendorf, Hauppauge, NY). The supernatant was collected in sample vials and stored at -4 °C for product and residue glucose analyses. Sugars were quantified by a binary HPLC system (Shimadzu Scientific Instruments, Columbia, MD) equipped with a refractive Index detector (RID-10A) and phenomenex RPM monosaccharide column (300×7.8 mm, Phenomenex, Torrance, CA). Deionised water was used as the mobile phase at a flow rate of 0.6 mL min^{-1}. The oven (Prominence CTD-20A) temperature was maintained at 80 °C. Lactic acids were quantified by a Chirex Chiral column (150×4.6 mm, Phenomenex, Torrance, CA) with isocratic 1 mmol L^{-1} copper (II) sulfate mobile phase at 1 mL min^{-1}. Peaks were monitored using a UV detector at 254 nm (Shimadzu, PDA).

Results and discussion

Enzymatic hydrolysis

Experiments with different loads of cellulase were performed to determine a suitable enzyme loading for enzymatic hydrolysis of pulp, modified pulp, and alkali-treated corn stover. The maximum reaction rate (v_{max}) was calculated from the Michaelis-Menten equation ($v = \frac{v_{max}[S]}{K_m + [S]}$). The v_{max} increased almost linearly with the increase of enzyme concentration in all three biomass cases (Fig. 3.1). The hydrolysis rate of corn stover and modified pulp was about to reach a plateau when the enzyme loading increased, perhaps due to substrate saturation

(Lee and Fan 1982). Increased enzyme loading from 2 to 8 FPU g-1 of substrate increased glucose yield by 24% after 48 h of pulp saccharification (Fig. 3.2A); however, increasing the enzyme dosage did not significantly change the final glucose yield in the saccharification of mechanically modified pulp (12 %) (Fig. 3.2B) and alkali treated corn stover (11 %) (Fig. 3.2C). The highest glucose yield was observed at 24 h for mechanically modified pulp as well as corn stover. The initial saccharification rate of mechanically modified pulp and corn stover was higher than that of pulp. Mechanically modified pulp had finer fiber size, which made it much easier for the enzymes to break down. Alkali treatment caused the cellulose in corn stover to swell, which led to an increase in the internal surface area and a decrease in the degree of crystallinity of cellulose (Chandra et al. 2011), therefore making cellulose in alkali-treated corn stover much easier for the enzyme to access.

Production of D-lactic acid by SHF

The purpose of this portion of the study was to produce D-lactic acid by *L. delbrueckii* using sugars derived from biomass as a cheap carbon source. We also tested another strain *Sporolactobacillus inulinus* ATCC 15538. Unlike in the results obtained by Fukushima et al. (2004), *S. inulinus* produced L-lactic acid instead of D-lactic acid in our experiments. This result may be due to the difference in strain or the possible alternation of bacterial character after receiving it.

In shake flask fermentation, the amount of pulp (1 g), mechanically modified pulp (1.3 g), and corn stover (1.2 g) was set up to obtain 10 g L^{-1} glucose after enzymatic hydrolysis. No residual glucose was observed after 30 h fermentation, and the final pH of the medium was between 5 to 5.5. The optical purity of D-lactic acid was 99.9 %. These results were in close agreement with Demirci and Pometto (1992). The highest yield of D-lactic acid was observed in corn stover hydrolysate (Table 3.1). Besides glucose, 5.6 g L^{-1} xylose and 1.7 g L^{-1}arabinose were also present in the corn stover hydrolysate; however, xylose remained unused, and arabinose was below detectable levels at the end of fermentation. *L. delbrueckii* cannot use xylose due to the lack of xylose isomerase and xylulokinase, two key enzymes in xylose assimilation (Okano et al. 2009b).

In fed-batch fermentation, almost all glucose was consumed within the first 36 h (first stage). In the second stage, feeding medium was added, and fermentation was completed within

80 h. The Luedeking-Piret equation ($\frac{1}{X}\frac{dP}{dt} = \alpha \frac{1}{X}\frac{dX}{dt} + \beta$) was used to describe the D-lactic acid production from synthetic medium in the first stage. Growth-associated constant (α) and non-growth associated constant (β) can be calculated from the graph of the specific production rate (q_p) versus the specific growth rate (μ); the correlation coefficient (R^2) was 0.88 (Fig. 3.3). Compared with other strains listed in Table 3.2, in our study *L. delbrueckii* had lower μ_{max} and higher α values. Lower μ_{max} suggests lower growth efficiency, and a high α value indicates a higher contribution of the cell growth to D-lactic acid production (Zhao et al. 2010). The value of α multiplied by μ_{max} was 1.56, which was larger than the β value, indicating that the specific growth rate played an important role in specific D-lactic acid production.

Fig. 3.4 and Fig. 3.5 show the fermentation profile of the synthetic medium and pulp hydrolysate, respectively. Table 3.3 summarizes the results of the first stage of fed-batch fermentation and batch fermentation. 37.4 g L^{-1} D-lactic acid was obtained in the end of first stage fermentation, and the product yield and productivity obtained in the first stage of fed-batch fermentation were 0.93 g g^{-1} and 1.04 g L^{-1} h^{-1}, respectively. These results were in agreement with that in other work (Vadlani et al. 2008a; Garde et al. 2002). Fed-batch fermentation was completed within 80 h; at the end of fermentation, about 5.5 g L^{-1} glucose was left and up to 57.3 g L^{-1} D-lactic acid with optical purity of 99.8 % was accumulated, which led to a productivity of 0.72 g L^{-1} h^{-1}. After pulp hydrolysis, the glucose concentration was 50 g L^{-1} and was used in the batch fermentation. After 30 h, glucose was hardly consumed, and even if we extended the fermentation time to 36 h, 6.2 g L^{-1} residual glucose remained. At the end of fermentation, 36.3 g L^{-1} lactic acid was produced, the yield of D-lactic acid was calculated by the amount of D-lactic acid produced divided by the amount of glucose consumed, which was 0.83 g g^{-1}, and productivity was 1.01 g L^{-1} h^{-1}. In a similar study undertaken in our laboratory, L-Lactic acid was synthesized from cheese whey. A yield of 0.98 g g^{-1} and productivity of 1.14 g L^{-1} h^{-1} was obtained (Vadlani et al. 2008a). The product formation rate of batch fermentation of pulp hydrolysate was quite close to the product formation rate of first-stage fed-batch fermentation using the synthetic medium. The yield of D-lactic acid (0.83 g g^{-1}) from pulp hydrolysate was lower than the first-stage yield (0.93 g g^{-1}) from synthetic medium. The reason might be due to substrate inhibition; therefore, the SSF process was preferred in subsequent experiments.

Production of D-lactic acid by SSF

After demonstrating the feasibility of producing D-lactic acid from biomass hydrolysate in the batch process, SSF was carried out using pulp and corn stover in a shake flask. In SSF, samples were collected after 4 h of incubation, the profiles obtained for corn stover and pulp SSF experiments are shown in Fig. 3.6. In SSF, cellulose hydrolysis and glucose assimilation were combined into a single fermentation process (Patel et al. 2006). During the first 8 h, bacteria were in low activity and glucose accumulated to around 8 g L^{-1} in the case of pulp and 14 g L^{-1} for corn stover. After the first 8 h cultivation, glucose concentration was kept low, which indicated that bacterial cells were metabolically active during the entire course of the fermentation and also meant that enzymatic hydrolysis of cellulose was the rate limiting step for D-lactic acid production as already observed by other groups (Parajo et al. 1997; Nakasaki and Adachi 2003). Xylose accumulated and remained nearly constant throughout the process. It was impossible to know the exact amount of glucose consumed in the SSF process, in order to compare SSF and SHF, results were expressed as an overall yield (the amount of D-lactic acid produced divided by the amount of biomass used) to compare SHF and SSF (Table 3.1). The highest D-lactic acid overall yield was 0.48 g g^{-1} of pulp in SSF and 0.38 g g^{-1} in SHF. For corn stover, the maximum D-lactic acid overall yield was 0.50 g g^{-1} in SSF and 0.41 g g^{-1} in SHF, demonstrating that the SSF process was more efficient than the SHF process. The reason for the higher overall yield in SSF may be that glucose released during the hydrolysis step was rapidly consumed as substrate during the fermentation step, therefore reducing the end-product inhibition of hydrolysis (Akerberg and Zacchi 2000).

Conclusions

In this study, we demonstrated efficient D-lactic acid production with high optical purity from pulp, modified pulp, and corn stover by *L. delbrueckii* ATCC 9649. Enzymatic hydrolysis of biomass was achieved effectively by CTec2 enzyme system. D-lactic acid productivity was not only high, but also cost-effective because pulp and modified pulp need no pretreatment. The SSF process demonstrated the advantages of avoiding substrate inhibition and increasing the productivity and yield of D-lactic acid. The yield obtained in the present study would have been even higher if xylose from corn stover hydrolysate could be completely used by the

microorganism. Future study should be directed toward complete use of the available carbohydrate for efficient D-lactic acid production.

Table 3.1 D-lactic acid production through SHF and SSF process in shake flask

		Initial glucose ($g\ L^{-1}$)	Lactic acid ($g\ L^{-1}$)	Yield[1] ($g\ g^{-1}$)	Overall yield[2] ($g\ g^{-1}$)	Productivity[3] ($g\ L^{-1}\ h^{-1}$)
SHF	Synthetic medium	10^{A}	7.7 ± 0.05^{A}	0.77 ± 0.01^{A}	---	0.25 ± 0.01^{A}
	Pulp	9.7 ± 0.17^{A}	7.5 ± 0.47^{A}	0.77 ± 0.66^{A}	0.38 ± 0.02^{A}	0.25 ± 0.03^{A}
	Modified pulp	11.2 ± 0.09^{B}	8.5 ± 0.39^{A}	0.76 ± 0.03^{A}	0.42 ± 0.02^{A}	0.28 ± 0.01^{A}
	Corn stover	9.9 ± 0.05^{A}	8.3 ± 0.04^{A}	0.83 ± 0.01^{A}	0.41 ± 0.01^{A}	0.27 ± 0.01^{A}
SSF	Pulp	---	19.2 ± 1.63^{B}	---	0.48 ± 0.04^{B}	0.31 ± 0.04^{A}
	Corn stover	---	20.1 ± 0.65^{B}	---	0.50 ± 0.03^{B}	0.32 ± 0.07^{A}

Data represent mean values and standard errors based on three replications ($p < 0.05$). Values with the same letters in the same column are not significantly different.

[1] Yield was calculated by the amount of D-lactic acid produced divided by the amount of glucose consumed.

[2] Overall yield was calculated by the amount of D-lactic acid produced divided by the amount of biomass used.

[3] Productivity was defined as the amount of D-lactic acid produced per liter per hour.

Table 3.2 Kinetic parameters of different lactic acid bacteria

Microorganism	Substrate	μ_{max} [1]	α	β	Reference
L. delbrueckii	Glucose	0.2	7.8	0.18	This study
L. lactis	Lactose	1.1	0.392	3.02	Boonmee et al. 2003
E. faecalis RKY1	Molasses	1.6	0.26	---	Nandasana and Kumar 2008
Lactobacillus helveticus	Whey permeate	0.48	2.33	0.77	Amrane 2005

[1] μ_{max} is the maximum specific growth rate (h^{-1})

Table 3.3 Kinetic parameters of fed-batch and batch fermentation

	Lactic acid ($g\ L^{-1}$)	Yield[1] ($g\ g^{-1}$)	Y_{PX} [2] ($g\ g^{-1}$)	Y_{XS} [3] ($g\ g^{-1}$)	q_{ps} [4] (h^{-1})	Productivity[5] ($g\ L^{-1}\ h^{-1}$)
Fed-batch (stage I)	37.4	0.93	10.9	0.086	0.026	1.04
Pulp hydrolysate batch	36.3	0.83	---	---	0.023	1.01

[1] Yield was calculated by the amount of D-lactic acid produced divided by the amount of glucose consumed.
[2] Y_{PX} was calculated by the amount of D-lactic acid produced divided by the amount of cell dry mass.
[3] Y_{XS} was calculated by the amount of cell dry mass divided by the amount of glucose consumed.
[4] q_{ps} is product formation rate, calculated based on the equation $q_{ps} = (1/S) \times (dP/dt)$
[5] Productivity was defined as the amount of D-lactic acid produced per liter per hour.

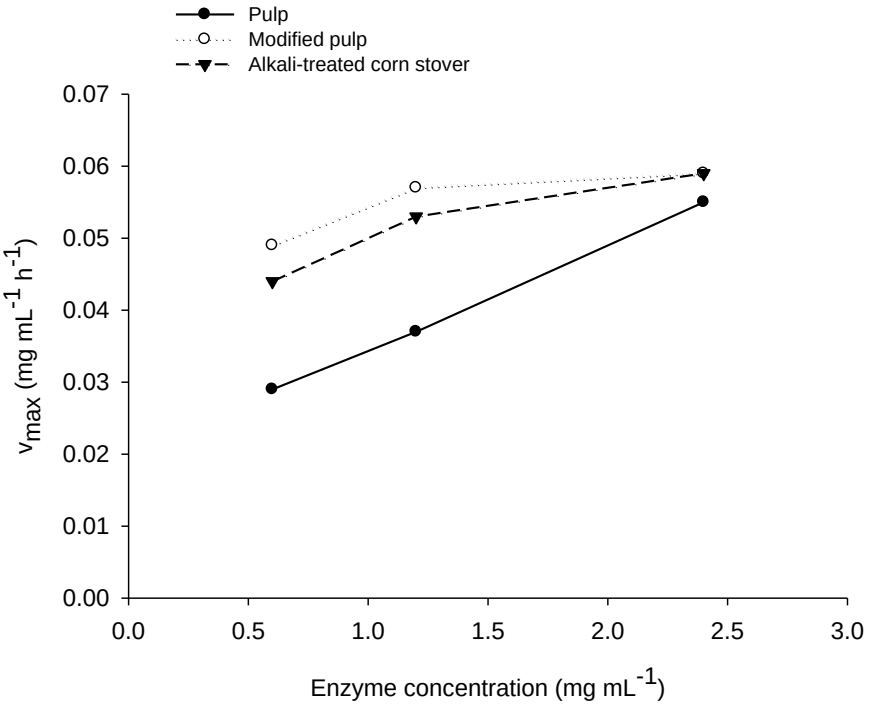

Figure 3.1 Plot of of v_{max} of different biomass versus enzyme concentration

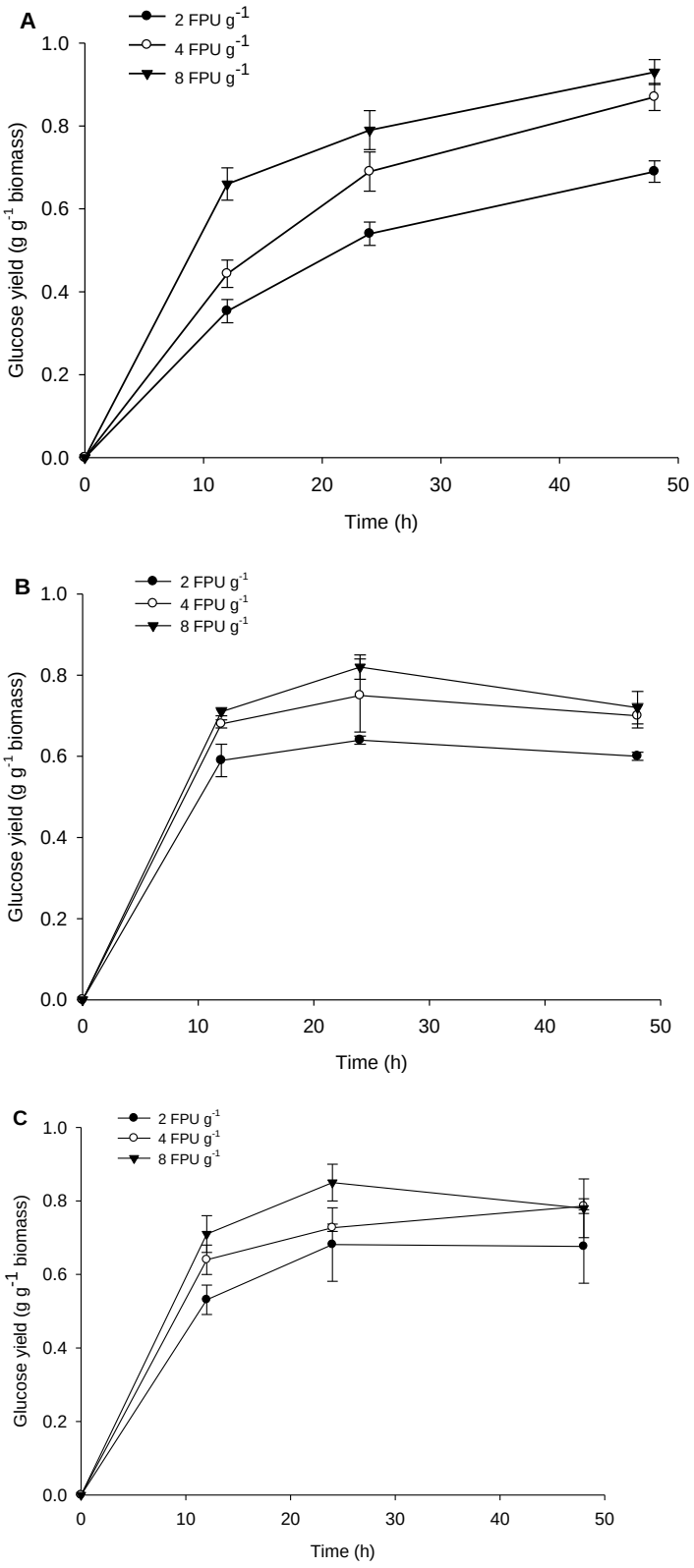

Figure 3.2 Enzymatic hydrolysis of (A) pulp; (B) mechanically modified pulp; and (C) alkali-treated corn stover at varying cellulase levels

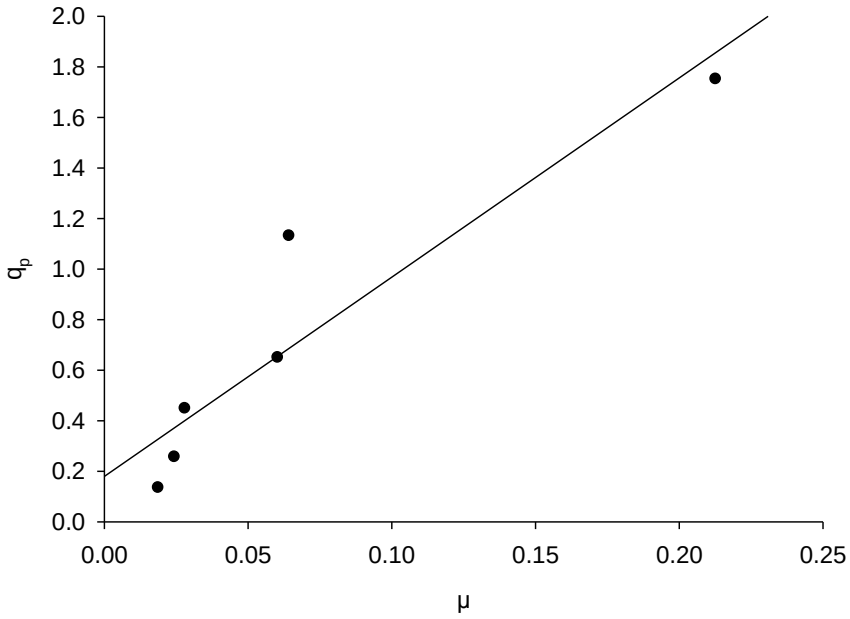

Figure 3.3 Specific production rate versus specific growth rate for *L.delbrueckii* growing on the synthetic medium

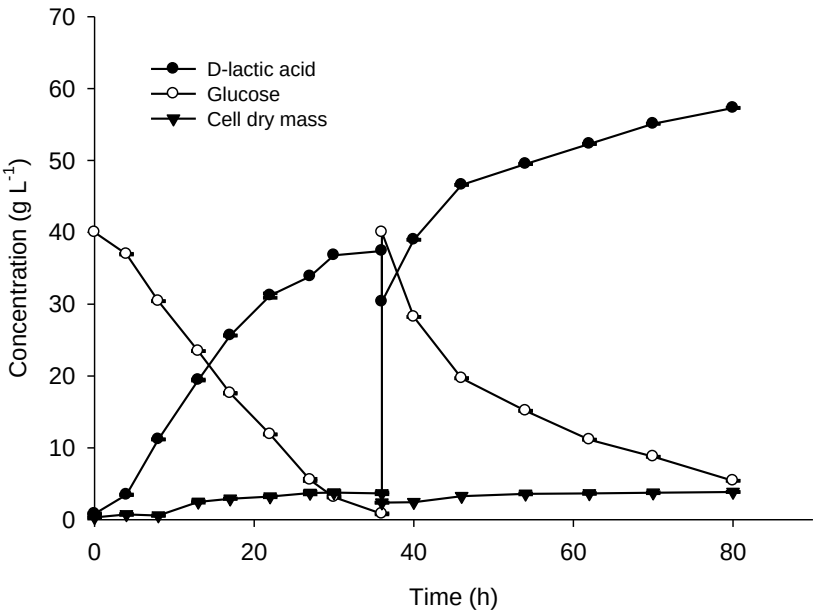

Figure 3.4 Fed-batch fermentation profile of D-lactic acid from the synthetic medium

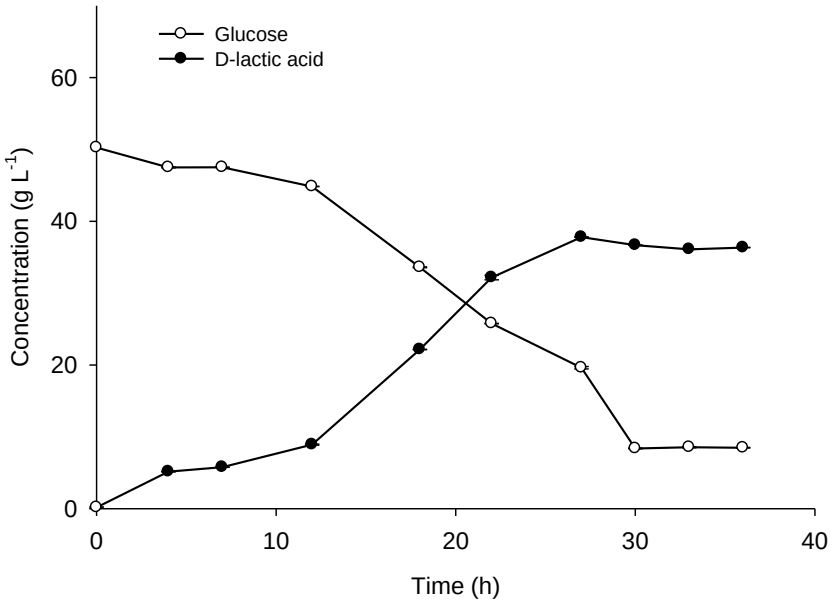

Figure 3.5 Batch fermentation profile of D-lactic acid production from pulp hydrolysate

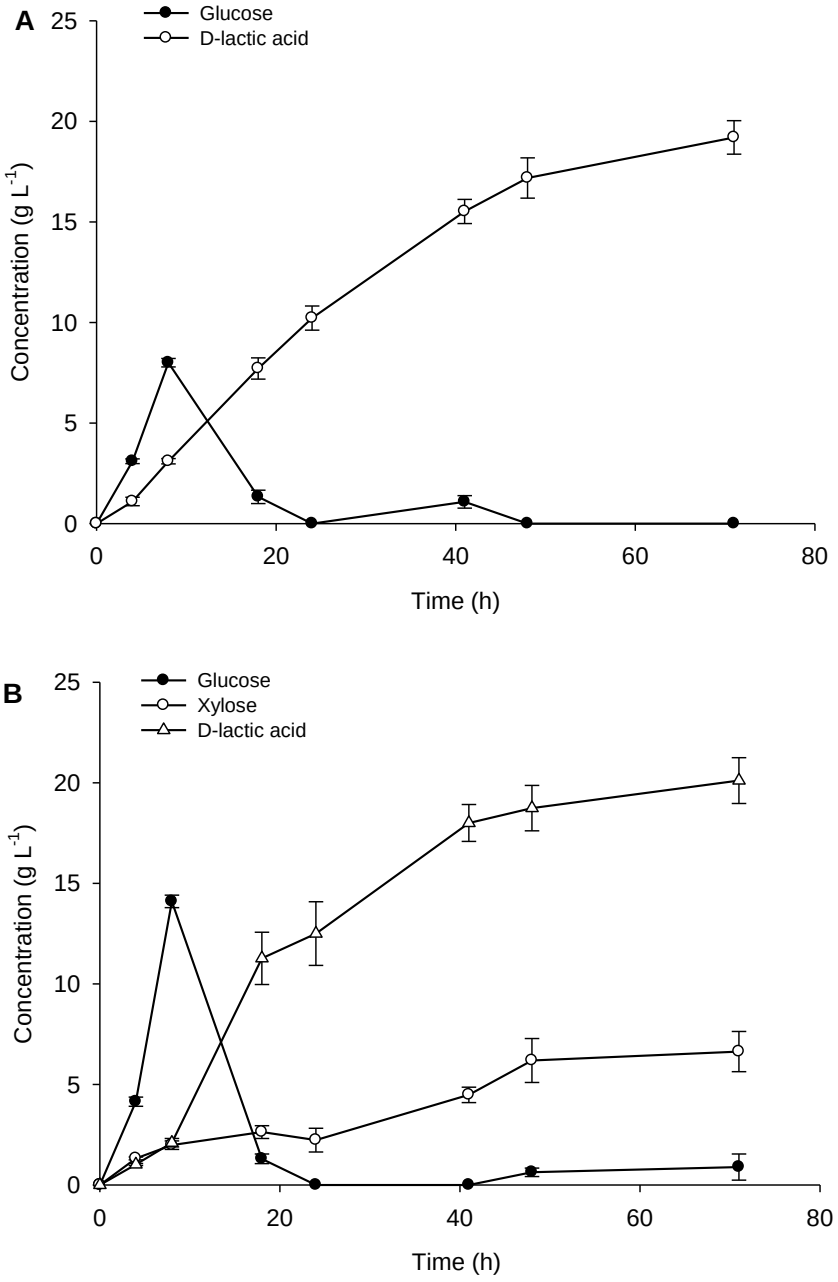

Figure 3.6 Time course of SSF process with *L. delbrueckii* using (A) pulp and (B) akali-treated corn stover

Chapter 4 - Lactic acid production from biomass-derived sugars via co-fermentation of *Lactobacillus brevis* and *Lactobacillus plantarum*[2]

Abstract

Lignocellulosic biomass is an attractive alternative resource for producing chemicals and fuels. Xylose is the dominating sugar after hydrolysis of hemicellulose in the biomass, but most microorganisms either cannot ferment xylose or have a hierarchical sugar utilization pattern in which glucose is consumed first. To overcome this barrier, *Lactobacillus brevis* ATCC 367 was selected to produce lactic acid. This strain possesses a relaxed carbon catabolite repression mechanism that can use glucose and xylose simultaneously; however, lactic acid yield was only 0.52 g g^{-1} from a mixture of glucose and xylose, and 5.1 g L^{-1} of acetic acid and 8.3 g L^{-1} of ethanol were also formed during production of lactic acid. The yield was significantly increased and ethanol production was significantly reduced if *L. brevis* was co-cultivated with *Lactobacillus plantarum* ATCC 21028. *L. plantarum* outcompeted *L. brevis* in glucose consumption, meaning that *L. brevis* was focused on converting xylose to lactic acid and the by-product, ethanol, was reduced due to less NADH generated in the fermentation system. Sequential co-fermentation of *L. brevis* and *L. plantarum* increased lactic acid yield to 0.80 g g^{-1} from poplar hydrolysate and increased yield to 0.78 g lactic acid per g of biomass from alkali-treated corn stover with minimum by-product formation. Efficient utilization of both cellulose and hemicellulose components of the biomass will improve overall lactic acid production and enable an economical process to produce biodegradable plastics.

Introduction

Lactic acid is a versatile chemical with a long history of applications in the food, cosmetic, and pharmaceutical industries (Yadav et al. 2011), and it has been listed as a platform chemical derived from biomass by the US Department of Energy since 2004 (Werpy and

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Petersen 2004). The estimated world demand for lactic acid will be 600,000 tons by 2020 (Dusselier et al. 2013) and is expected to keep increasing because of their use in the development of poly-lactic acid (PLA) and lactate solvents (Hofvendahl and Hahn-Hagerdal 2000).

Biomass-based fermentation products have gained intensive attention recently due to their potential as fossil fuel substitutes. More than 90 % of global production of plant biomass is lignocellulose, which is mainly composed of cellulose, hemicellulose, and lignin (Yang et al. 2009). Total cellulose and hemicellulose content is higher in hardwood (78.8 %) than in softwood (70.3 %), but lignin content is opposite (Balat 2009). Various pretreatment methods have been developed, such as alkali treatment and ammonia explosion, to convert structural carbohydrates to monomer sugars (Kumar et al. 2009). Xylose is the dominant sugar released from hemicellulose in hardwoods and agricultural residues (Taherzadeh and Karimi. 2008). Efficient utilization of all sugars derived from biomass has the potential to reduce the production cost of chemicals by about 25 % (Hinman et al. 1989). Most homofermentative lactic acid bacteria, including *Lacbobacillus delbrueckii* (Fukushima et al. 2004; Zhang and Vadlani 2013), *Lactobacillus paracasei* (Moon et al. 2012b), and *Lactobacillus lactis* (Kosugi et al. 2010) cannot xylose to lactic acid. In contrast, some heterofermentative lactic acid bacteria, such as *Lactobacillus brevis* CHCC 2097 and *Lactobacillus pentosus* CHCC 2355, have been used to produce lactic acid from xylose released from wheat straw (Garde et al. 2002). These heterofermentative strains also produce considerable amounts of byproducts, such as acetic acid and ethanol, which increase product cost and decrease productivity (Abdel-Rahman et al. 2011). A third group of lactic acid bacteria known as the facultative heterofermenters, for example *Lactobacillus plantarum*, they use glucose through the Embden-Meyerhof pathway (EMP) to produce lactic acid, while they may also possess an inducible phosphoketolase pathway (PK) with pentose acting as inducers (Fugelsang and Edwards 2007).

Vadlani et al. (2008a) reported using *L. plantarum* ATCC 21028 in the first stage of fermentation to produce lactate from cheese whey; a product yield of 0.98 g lactate per g of lactose was obtained. Fu and Mathews (1999) also reported the kinetic model of lactic acid production by *L. plantarum* ATCC 21028 and found out that lactic acid fermentation with this bacterium is homolactic and primary growth associated. *Lactobacillus brevis,* a well-known heterofermentative strain, was reported to use xylose simultaneously with glucose (Kim et al. 2009), which is highly desirable because the strain does not possess carbon catabolite repression

(CCR), especially when operating under simultaneous saccharification and fermentation (SSF) conditions (Kim et al. 2010). In SSF, the glucose consumption rate needs to be higher than the release rate to ensure that no glucose remains in the medium; otherwise, xylose will not be used by microorganisms that have a hierarchical sugar utilization pattern.

Literature on the production of lactic acid through co-fermentation systems is limited (Garde et al. 2002; Cui et al. 2011; Nancib et al. 2009; Taniguchi et al. 2004),). Cui et al. (2011) reported co-cultivation of *L. rhamnosus* and *L. brevis* to produce lactic acid from corn stover with a yield of 0.7 g g^{-1}. To the best of our knowledge, no report discusses co-cultivation of *L. brevis* with *L. plantarum* from lignocellulosic biomass. We hypothesized that *L. plantarum* converts most glucose to lactic acid using the EMP, whereas *L. brevis* converts xylose and a small portion of glucose to lactic acid and acetic acid *via* the phosphoketolase pathway (Fig. 4.1). In this case, both glucose and xylose derived from lignocellulosic biomass can be used efficiently, and *L. brevis* can focus on converting xylose to lactic acid only because available glucose is limited; the by-product ethanol, which is produced mainly when glucose concentration is high, can be reduced due to glucose depletion by *L. plantarum*. The objective of this study was to evaluate the performance of mixed cultures of *L. plantarum* and *L. brevis* and to utilize poplar hydrolysate and corn stover, two promising biomasses representing hardwood and agriculture residues, respectively, as feedstocks to produce lactic acid.

Materials and methods

Microorganism and culture conditions

Lactobacillus brevis ATCC 367 and *Lactobacillus plantarum* ATCC 21028 obtained from the American Type Culture Collection (Manassas, VA, USA) were used in this work. *L. brevis* and *L. plantarum* inocula were grown in 50 mL liquid MRS medium (MRS broth, Difco Laboratories, Detroit, MI, USA) and incubated at 37 °C for 15 h at an agitation rate of 150 rpm in a temperature-controlled shaker (Innova 4300, New Brunswick Scientific, NJ, USA). N_2 was sparged into the bottle to create anaerobic growing conditions. The initial cell density of *L. brevis* and *L. plantarum* were measured using colony forming unit (CFU) counting method, the overnight cultures were diluted to different concentrations, plated on MRS agar and incubated at 37 °C for 24 h. Colonies were counted using a colony counter (Lab-Aids Inc., NY, USA). Initial cell concentrations of *L .plantarum* and *L. brevis* were 3×10^9, and 1.2×10^{10} CFU/mL,

respectively, which were adjusted to approximately 10^9 CFU/mL for inoculation in all fermentation experiments.

Poplar hydrolysate was obtained from Technology Holding LLC (Salt lake city, UT, USA), which contained 415 g L^{-1} of glucose, 132 g L^{-1} of xylose and 13 g L^{-1} of cellobiose. The initial pH of poplar hydrolysate was 2.6, which was adjusted to 6.5 using 10 mol L^{-1} of NaOH.

Corn stover was harvested from the Kansas State University Agronomy Farm in Manhattan and Tribune, Kansas, USA. Corn stover was treated with 1 % NaOH (w/v) using the method described by Guragain et al. (2013). The alkali-treated corn stover was dried at 60˚C overnight and ground to particle size of <1 mm using a laboratory mill (3303, Perten Instruments, Springfield, IL, USA).

Fermentation

Shake flask fermentation was performed in 100 mL Wheaton serum bottles containing 50 mL of modified MRS medium or poplar hydrolysate. The modified MRS medium consisted of glucose and xylose in a 3:1 ratio and supplemented with 10 g L^{-1} of peptone, 5 g L^{-1} of yeast extract, 2 g L^{-1} of ammonium citrate, 2 g L^{-1} of sodium acetate, 2 g L^{-1} of K_2HPO_4, 0.1 g L^{-1} of $MgSO_4.7H_2O$, 0.05 g L^{-1} of $MnSO_4.4H_2O$, and 1 g L^{-1} of Tween 80. Poplar hydrolysate was diluted and supplemented with all the components (except glucose and xylose) of the modified MRS medium. The pH of the medium was adjusted to 6.5 using 10 mol L^{-1} NaOH, and 3 % (w/v) of calcium carbonate was added to control the pH. Temperature was maintained at 37 °C for both *L. plantarum* and *L. brevis.* Agitation was maintained at 150 rpm.

In the simultaneous co-culture fermentation experiment, *L. plantarum* and *L. brevis* were inoculated at the same time at the beginning of fermentation with 5 % (v/v) inoculum for each strain at a 1:1 ratio. In the sequential fermentation test, 5 % (v/v) *L. plantarum* was added first, then 5 % (v/v) of *L. brevis* was inoculated when glucose concentration reached around 5 g L^{-1}.

Batch fermentation was performed in a 7-L fermenter with a working volume of 5 L (Bioflo 110, New Brunswick Scientific Inc., Enfield, CT, USA). The fermentation broth consisted of 1L diluted poplar hydrolysate (190 g L^{-1} glucose, 72 g L^{-1} of xylose and 6 g L^{-1} of cellobiose), 2 g L^{-1} of ammonium citrate, 2 g L^{-1} of sodium acetate, 2 g L^{-1} of K_2HPO_4, 0.1 g L^{-1} of $MgSO_4.7H_2O$, 0.05 g L^{-1} of $MnSO_4.4H_2O$, and 1 g L^{-1} of Tween 80. During fermentation, the

temperature was maintained at 37 ºC; agitation speed at 100 rpm; and pH at 6.5 by adding 10 mol L^{-1} NaOH. N_2 was sparged at 0.6 vvm through the vessel to maintain anaerobic conditions.

Simultaneous saccharification and fermentation (SSF) was conducted in 100-mL serum bottles. 4 % (w/v) pretreated corn stover was suspended in 50 mL of 0.05 mol L^{-1} sodium citrate buffer supplemented with all the components except sugars of the modified MRS medium. Cellic CTec2 (CTec2) obtained from Novozymes Inc. (Franklinton, NC, USA) was added at 8 FPU/g of biomass. Temperature was maintained at 37 ºC, and the agitation rate was maintained at 150 rpm.

Analytical methods

Glucose, xylose, and lactic acid were measured according to the method described by Zhang and Vadlani (2013). Acetic acid and ethanol were measured using high-performance liquid chromatography (HPLC; Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) equipped with a Rezex ROA organic acid column (150 × 7.8 mm, Phenomenex Inc., Torrance, CA, USA) and a refractive index (RI) detector (RID-10A). 0.005 N H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL min^{-1}. Temperatures of the column and detector were maintained at 83 and 40 °C, respectively.

Statistical methods

SAS software version 9.1 (SAS Inc. Cary, NC) was used to analyze experimental data by applying PROC GLM.

Results and discussion

Lactic acid production from a mixture of glucose and xylose

The theoretical *L. plantarum* yield of lactic acid from glucose via the EMP is 1 (g per g of glucose). Fig. 4.2A shows fermentation profile of *L. plantarum* from a mixture of glucose and xylose. *L. plantarum* consumed glucose rapidly; only 4.4 g L^{-1} glucose was left at 12 h, and it was completely consumed within 24 h of fermentation. 24.3 g L^{-1} of lactic acid was obtained from 25.5 g L^{-1} of glucose with a D/L lactic acid molar ratio of 0.94 (48.5 % of optical purity) .

L. brevis can use both glucose and xylose via the PK pathway and produces a mixture of lactic acid, acetic acid, and ethanol. The theoretical yield of lactic acid from glucose and xylose

is 0.5 g per g of glucose and 0.6 g per g of xylose. As shown in Fig. 4.2B, *L. brevis* barely consumed glucose and xylose in the first 8 h of fermentation, after which glucose was consumed much faster than xylose; all glucose was consumed after 24 h, and xylose was completely used after 48 h. Final lactic acid concentration was 17.2 g L^{-1}, acetic acid concentration was 5.1 g L^{-1}, and ethanol concentration was 8.3 g L^{-1}. The D/L molar ratio of lactic acid was 0.37 (27.0 % of optical purity). Kim et al (2009) investigated the proteome of *L. brevis* grown in glucose, xylose, and a glucose/xylose mixture. The relative expression of a putative acetate kinase was expressed at a much higher level when cells were grown in xylose as single carbon source, which resulted in a different end-product profile. *L. brevis* in our study also showed a different end-product profile when a different carbon source was used. The acetate/ethanol molar ratio was 0.1 when glucose was the sole carbon source, but the molar ratio changed to 2.5 when xylose was used as the only carbon source. The ratio of acetate/ethanol depends on the oxidation reduction potential ($NADH/NAD^+$ ratio) of the fermentation system (Kandler 1983). NADH is required for ethanol and lactic acid production. More NADH is generated from glucose catabolism than from xylose metabolism; therefore, acetaldehyde is converted to ethanol coupled with the regeneration of NAD^+ from NADH (Fig. 4.1).

Unlike a single culture of *L. brevis*, simultaneous fermentation of *L. brevis* and *L. plantarum* did not exhibit a sugar consumption lag phase in the first 8 h of fermentation. Glucose was consumed within 24 h, and xylose was consumed within 48 h (Fig. 4.2C). Final lactic acid concentration increased to 28.3 g L^{-1} with a D/L molar ratio of 0.61 (37.8% of optical purity), and ethanol decreased to 2.1 g L^{-1} (Table 4.1). The maximum glucose consumption rate of *L. plantarum* was almost 5 times greater than that of *L. brevis*, which suggests that *L. plantarum* outcompetes *L. brevis* for glucose consumption when these two strains grow together. Furthermore, the ethanol production by *L. brevis* was inhibited due to glucose depletion by *L. plantarum*.

The fermentation profile of sequential co-fermentation of *L. brevis* with *L. plantarum* is shown in Fig. 4.2D. Different inoculation times of *L. brevis* were tested; no ethanol was produced when *L .brevis* was inoculated at glucose concentration around 5 g L^{-1}. 30.5 g L^{-1} of lactic acid was obtained by the end of fermentation, with a D/L molar ratio of 0.65 (39.4 % of optical purity). If all glucose entered the EMP pathway, and if all xylose entered the PK

pathway, the theoretical production of lactic acid was: Lactic acid (g L^{-1}) = (glucose (g) ×1+xylose (g) ×0.6)/volume (L).

Simultaneous and sequential fermentation of *L. plantarum* and *L. brevis* increased lactic acid production efficiency to 89 % and 95 % of the theoretical maximum production, respectively.

Taniguchi et al (2004) also reported highest concentration of lactic acid (95 g L^{-1}) with a mixed culture system of *Lactobacillus casei* and *Enterococcus casseliflavus* from a mixture of glucose (100 g L^{-1}) and xylose (50 g L^{-1}). While simultaneous inoculation of *L. casei* and *E. casseliflavus* did not increase the lactic acid production, sequentially inoculated *E. casseliflavus* after 40 h allowed completely consumption of xylose and enhanced final lactic acid concentration at the expense of lactic acid productivity (0.49 g L^{-1} h^{-1}). Compared with the two-stage system reported by Taniguchi, the co-cultivation system in this study has higher lactic acid productivity (0.59 g^{-1} L^{-1} h^{-1}) and higher lactic acid yield (0.85 g g^{-1}).

Lactic acid production from poplar hydrolysate

The poplar hydrolysate was detoxified by the company and delivered to us; hence, no inhibition of cell growth was observed in our experiments. Table 4.2 summarizes the fermentation results. *L. plantarum* produced 25.6 g L^{-1} of lactic acid from 29.7 g L^{-1} glucose. The D/L lactic acid molar ratio was 0.98 (49.5 % of optical purity), which is very close to that obtained from synthetic sugars. *L. brevis* produced 18.8 g L^{-1} lactic acid from 29.6 g L^{-1} of glucose, and 9.4 g L^{-1} of xylose with a D/L molar ratio of 1.2 (54.5 % of optical purity). 4.5 g L^{-1} acetic acid and 11.5 g L^{-1} ethanol were also obtained. Compared with the simultaneous fermentation experiment, sequential fermentation of *L. plantarum* and *L. brevis* increased lactic acid yield from 0.71 to 0.80 g g^{-1} and increased lactic acid production efficiency from 79 % to 88 % of the theoretical maximum production. The D/L lactic acid molar ratio was 1 (50.0 % of optical purity) for both simultaneous and sequential co-fermentation. Statistically, sequential fermentation gave the highest product concentration and yield at the expense of relatively lower productivity. Consequently, sequential fermentation was scaled up to a 7-L fermenter with 5-L working volume using poplar hydrolysate (Fig. 4.3). Initial glucose and xylose concentrations were 35.4 g L^{-1} and 14.3 g L^{-1}, respectively. *L. brevis* was added at 20 h when glucose concentration was 5.2 g L^{-1}; glucose was completely consumed within 26 h. 2.2 g L^{-1} of xylose

was left after 96 h of fermentation. Final lactic acid concentration was 38.0 g L^{-1} with 7.2 g L^{-1} acetic acid, and no ethanol was detected. The yield of lactic acid was 0.80 g g^{-1}, and productivity was 0.40 g L^{-1} h^{-1}.

Garde et al. (2002) evaluated lactic acid production from hemicellulose of wheat straw hydrolysate by single or mixed culture of *Lactobacillus pentosus* and *Lactobacillus brevis*. The mixed culture system increased lactic acid production efficiency to 95 % of the theoretical maximum yield. Nancib et al. (2009) also reported lactic acid production from date juice extract by a mixed culture system of *Lactobacillus casei* and *Lactobacillus lactis*, which gave better lactic acid production and sugar utilizations. All these results corroborate the results we found that mixed cultures of lactic acid bacteria are more efficient than single culture regarding lactic acid concentration and sugar utilizations.

Lactic acid production from corn stover via SSF

Corn stover is the most abundant agriculture residue in U.S, with annual production of 105–117 million dry tons (Graham et al. 2007). Alkali-treated corn stover consisted of around 54 % (w/w) glucan, 29 % (w/w) xylan and a small amount of arabinan (Guragain et al. 2013). The theoretical sugar yields from 2 g of dried alkali-treated corn stover were 1.2 g glucose, and 0.7 g xylose. Temperature and pH can be set at optimal conditions for either the enzyme or the bacteria. In this study, optimal growth temperature of bacteria was determined by measuring the optical density at 600 nm under 30, 37, 40 and 45 ℃. Both culture grew best at 37 ℃. The temperature was set to the optimum for the bacteria, because bacterial growth was significantly reduced under the optimal temperature range of CTec2 (45–50 ℃), whereas the CTec2 still retains 60 % of its hydrolysis activity compared to hydrolysis conducted under optimal conditions. The initial pH was set at 6, which between the optimal pH for enzymes and bacteria, and the relative performance of enzymes decreases only 10 % under this pH according to the Novozymes application sheet (Luna No. 2010-01668-01). Fig. 4.4A shows lactic acid production from corn stover from a single culture of *L. plantarum*. Glucose released from corn stover was consumed rapidly by *L. plantarum;* the concentration of glucose was maintained at a low level throughout the SSF process, which suggests that hydrolysis was the rate-limiting step. *L. plantarum* in this study cannot use xylose, but it was able to use arabinose at very slow rate and produced lactic acid (yield of 0.38 g lactic acid per g of arabinose) and trace amount of

acetic acid (yield of 0.07 g acetic acid per g of arabinose). Arabinose was not counted into lactic acid production in this study due to the small amount of arabinose (less than 1.5 g L^{-1}) present in the corn stover hydrolysate. Fig. 4.4B shows *L. brevis* performance for lactic acid production from corn stover, and *L. brevis* consumed glucose faster than xylose. Glucose concentration reached a maximum level of 5.9 g L^{-1} at 12 h, then rapidly decreased to 0.9 g L^{-1} in the next 12 h, and no glucose was detected at 48 h. Xylose increased to 4.4 g L^{-1} at 6 h, then slowly decreased to 0.7 g L^{-1} during the remaining time of fermentation. The final concentration of lactic acid was 16.3 g L^{-1}, acetic acid concentration was 5.1 g L^{-1} and ethanol concentration was 10.0 g L^{-1}. Fig. 4.4C shows lactic acid production by simultaneous fermentation of *L. plantarum* and *L. brevis*. Glucose and xylose accumulated to the maximum level of 1.6 g L^{-1} and 4.4 g L^{-1} at 6 h, respectively. Glucose concentration decreased to 0.2 g L^{-1} at 12 h and remained at zero during the duration of fermentation. Xylose concentration decreased to 1.9 g L^{-1} at 24 h, and no xylose was detected during the next 24 h. 24.0 g L^{-1} of lactic acid, 6.2 g L^{-1} of acetic acid, and 1.2 g L^{-1} of ethanol were obtained at the end of fermentation. In sequential fermentation, as shown in Fig. 4.4D, *L. brevis* was added when xylose concentration reached 8.2 g L^{-1} at 24 h, which was 61 % of the theoretical hydrolysis yield of xylose from 1 % sodium hydroxide treated corn stover. In the first stage, glucose released from corn stover was quickly consumed by *L. plantarum* to produce lactic acid. In the second stage, accumulated xylose was consumed by *L. brevis*, and *L. plantarum* kept consuming glucose. Lactic acid increased to 31.2 g L^{-1}, and 6.3 g L^{-1} of acetic acid was obtained after 72 h of fermentation.

The performance of co-cultivation of *L. plantarum* and *L. brevis* was better than the individual strain during fermentation (Table 4.3). The highest lactic acid concentration (31.2 g L^{-1}) and overall yield (0.78 g g^{-1}) were obtained in sequential fermentation, whereas the highest productivity (0.50 g L h^{-1}) was obtained in simultaneous fermentation, with an overall yield of 0.57 g g^{-1}. In sequential fermentation, the overall yield was higher, whereas productivity was lower than that reported by Cui et al. (2011), which were 0.70 g g^{-1} and 0.58 g L^{-1} h^{-1}, respectively, in the fermentation of alkali-treated corn stover with mixed cultures of *L. rhamnosus* and *L. brevis*. The lower productivity in our study is mainly attributed to the lower enzyme dosage (8 FPU/g) compared with that (25 FPU/g) used in Cui's study; consequently, the total process time was elongated.

Conclusions

In conclusion, the novel co-fermentation system in this study took advantage of both *lactobacillus* strains and enabled optimum utilization of sugars derived from lignocellulosic biomass. This mixed culture system showed better sugar utilization, enhanced lactic acid production, and formed minimal by-products, especially when operated in SSF mode. Metabolic flow of sugars in this co-cultivation system need to be investigated in detail to further increase lactic acid yield and decrease by-product formation. Because the process is greatly simplified by the similar cultivation conditions of these two strains, the co-cultivation system has enormous potential for industrial applications. In addition, optimal conditions such as inoculum size, temperature, and substrate concentration in the SSF process can be found with the help of response surface methodology.

Table 4.1 Lactic acid production by single or mixed culture of *L. brevis* and *L. plantarum* from a mixture of glucose and xylose

	Glucose ($g\ L^{-1}$)	Xylose ($g\ L^{-1}$)	Lactic acid ($g\ L^{-1}$)	Acetic acid ($g\ L^{-1}$)	Ethanol ($g\ L^{-1}$)	Yield [1] ($g\ g^{-1}$)	Productivity [2] ($g\ L^{-1}h^{-1}$)	$r_{s,glu}$ [3] ($g\ L^{-1}h^{-1}$)	Optical purity [4] (%)
L. plantarum	25.5 ± 1.1^{A}	8.6 ± 0.2^{A}	24.3 ± 0.4^{B}	0^{B}	0^{C}	0.96 ± 0.04^{A}	1.01 ± 0.02^{A}	2.9	48.5
L. brevis	25.0 ± 0.5^{A}	8.8 ± 0.2^{A}	17.2 ± 0.5^{C}	5.1 ± 0.4^{B}	8.3 ± 0.1^{A}	0.52 ± 0.02^{C}	0.36 ± 0.01^{D}	0.6	27.0
Simultaneous	26.4 ± 0.2^{A}	8.8 ± 0.1^{A}	28.3 ± 0.2^{A}	4.7 ± 0.4^{B}	2.1 ± 0.0^{B}	0.80 ± 0.01^{B}	0.59 ± 0.00^{B}	---	37.8
Sequential	27.0 ± 0.0^{A}	9.0 ± 0.0^{A}	30.5 ± 0.9^{A}	4.9 ± 0.4^{B}	0^{C}	0.85 ± 0.02^{B}	0.51 ± 0.02^{C}	---	39.4

Data represent mean values and standard errors based on three replications ($p < 0.05$). Values with the same letters in the same column are not significantly different.

[1] Lactic acid yield was calculated by dividing the amount of lactic acid by the amount of sugar consumed.

[2] Productivity was defined as the amount of lactic acid produced per liter per hour.

[3] Maximum glucose consumption rate calculated based on the equation $r_{s,glu} = q_s X$.

[4] Optical purity (OP) calculated based on the equation: OP=100×(D-lactic acid concentration)/(Total lactic acid concentration)

Table 4.2 Lactic acid production by a single or mixed culture of *L. brevis* and *L. plantarum* from poplar hydrolysate

	Glucose ($g\ L^{-1}$)	Xylose ($g\ L^{-1}$)	Lactic acid ($g\ L^{-1}$)	Acetic acid ($g\ L^{-1}$)	Ethanol ($g\ L^{-1}$)	Yield [1] ($g\ g^{-1}$)	Productivity [2] ($g\ L^{-1}h^{-1}$)	Optical purity [3] (%)
L. plantarum	29.7 ± 0.6^{A}	9.4 ± 0.2^{A}	25.6 ± 1.1^{C}	0^{C}	0^{C}	0.87 ± 0.03^{A}	1.08 ± 0.05^{A}	49.5
L. brevis	29.6 ± 0.6^{A}	9.4 ± 0.3^{A}	18.8 ± 0.7^{D}	4.5 ± 0.3^{B}	11.5 ± 0.7^{A}	0.48 ± 0.02^{C}	0.31 ± 0.01^{D}	54.5
Simultaneous	29.3 ± 0.6^{A}	10.2 ± 0.3^{A}	28.1 ± 0.6^{B}	5.3 ± 0.5^{A}	2.9 ± 0.3^{B}	0.71 ± 0.01^{B}	0.43 ± 0.01^{C}	50.0
Sequential	30.1 ± 0.1^{A}	10.1 ± 0.2^{A}	31.8 ± 0.1^{A}	5.6 ± 0.2^{A}	0^{C}	0.80 ± 0.01^{A}	0.48 ± 0.00^{B}	50.0

Data represent mean values and standard errors based on three replications ($p < 0.05$). Values with the same letters in the same column are not significantly different.

[1] Lactic acid yield was calculated by dividing the amount of lactic acid by the amount of sugar consumed.

[2] Productivity was defined as the amount of lactic acid produced per liter per hour.

[3] Optical purity (OP) calculated based on the equation: OP=100×(D-lactic acid concentration)/(Total lactic acid concentration)

Table 4.3 Lactic acid production by a single or mixed culture of *L. brevis* and *L. plantarum* from NaOH-treated corn stover

	Lactic acid ($g\ L^{-1}$)	Acetic acid ($g\ L^{-1}$)	Ethanol ($g\ L^{-1}$)	Overall yield [1] ($g\ g^{-1}$)	Productivity [2] ($g\ L^{-1}h^{-1}$)	Optical purity [3] (%)
L. plantarum	21.0 ± 0.3^{C}	0^{B}	0^{C}	0.50 ± 0.03^{C}	0.44 ± 0.01^{B}	42.2
L. brevis	16.3 ± 0.2^{D}	5.1 ± 0.2^{A}	10.0 ± 0.3^{A}	0.39 ± 0.01^{D}	0.34 ± 0.00^{C}	39.7
Simultaneous	24.0 ± 0.6^{B}	6.2 ± 0.1^{A}	1.2 ± 0.0^{B}	0.57 ± 0.01^{B}	0.50 ± 0.01^{A}	47.1
Sequential	31.2 ± 0.3^{A}	6.3 ± 0.6^{A}	0^{C}	0.78 ± 0.08^{A}	0.43 ± 0.05^{B}	43.2

Data represent mean values and standard errors based on three replications ($p < 0.05$). Values with the same letters in the same column are not significantly different.

[1] Lactic acid overall yield was calculated by dividing the amount of lactic acid produced by the amount of biomass consumed.

[2] Productivity was defined as the amount of lactic acid produced per liter per hour.

[3] Optical purity (OP) calculated based on the equation: OP=100×(D-lactic acid concentration)/(Total lactic acid concentration)

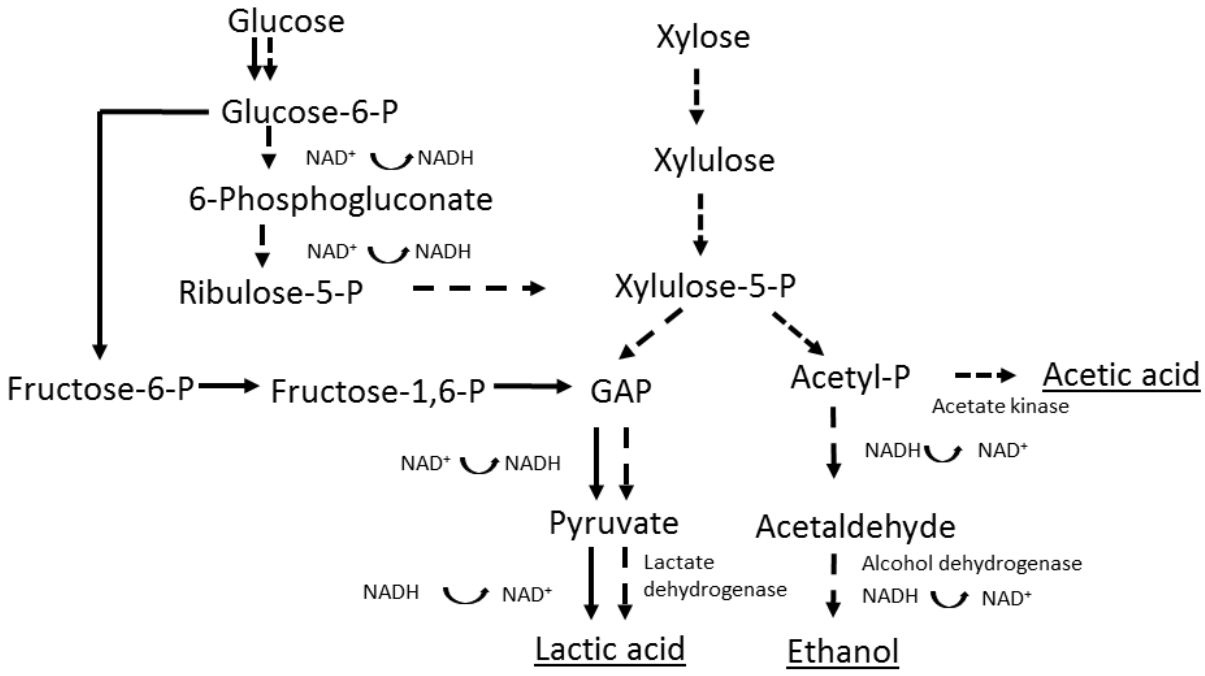
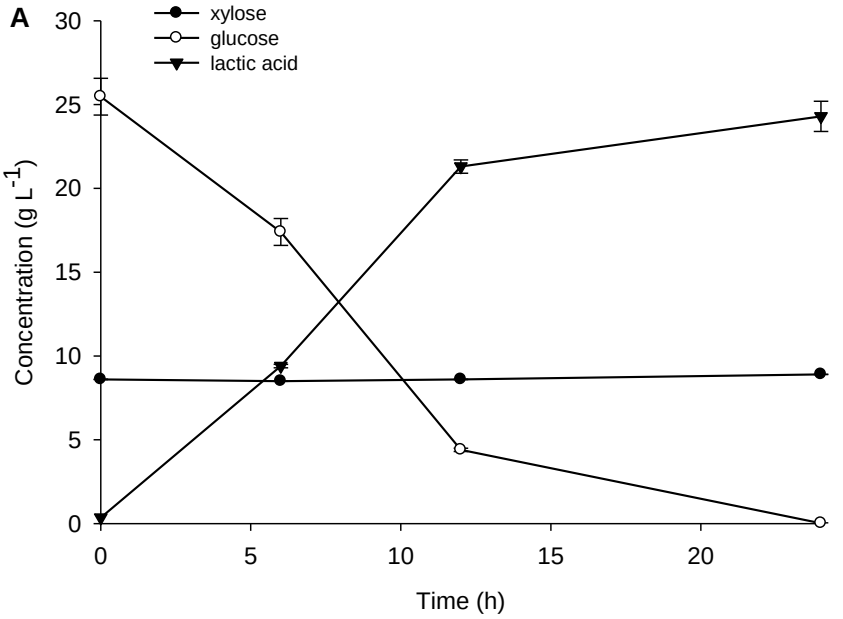
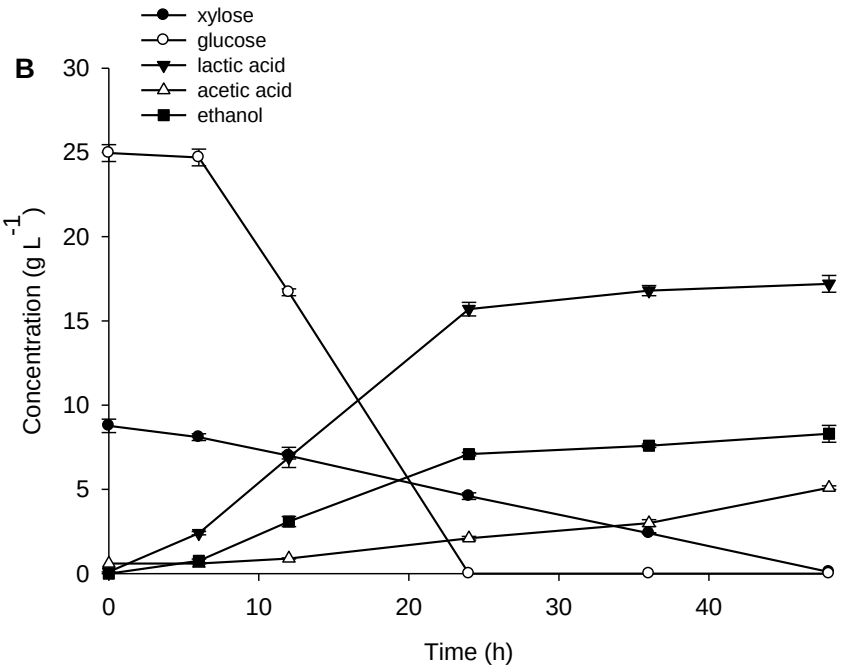


Figure 4.1 Simplified pathways for lactic acid production from a mixture of glucose and xylose by *L. plantarum* and *L. brevis*. Solid lines indicate the homofermentative pathway in *L. plantarum*, and dashed lines indicate the heterofermentative pathway in *L. brevis*


A
xylose
glucose
lactic acid
Concentration (g L-1)
Time (h)
0
5
10
15
20
25
30


B
xylose
glucose
lactic acid
acetic acid
ethanol
Concentration (g L-1)
Time (h)
0
10
20
30
40

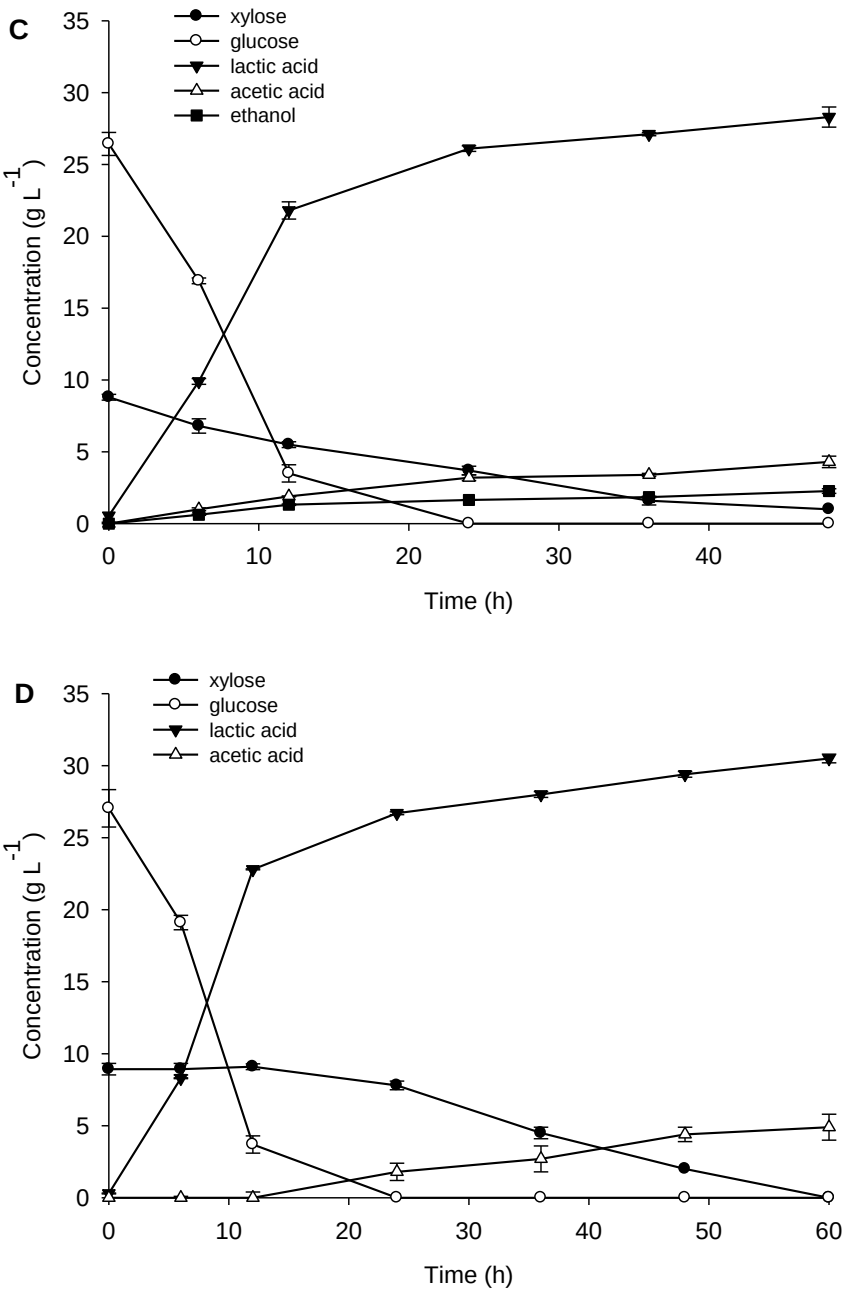


Figure 4.2 Lactic acid production from a mixture of glucose and xylose by (A) *L. plantarum*; (B) *L. brevis*; (C) simultaneous fermentation of *L. plantarum* and *L. brevis*; and (D) sequential fermentation of *L. plantrum* and *L. brevis*

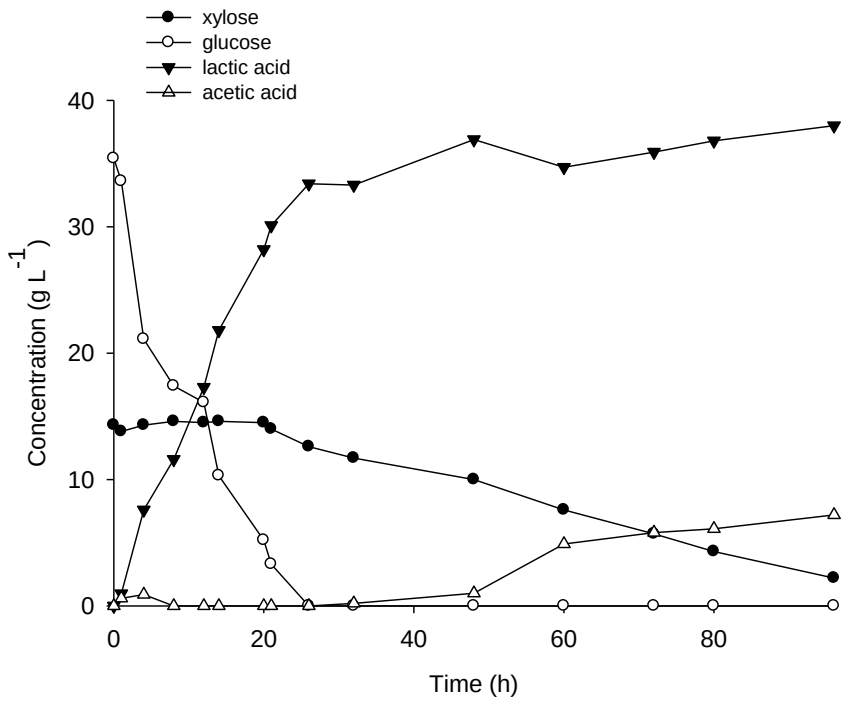
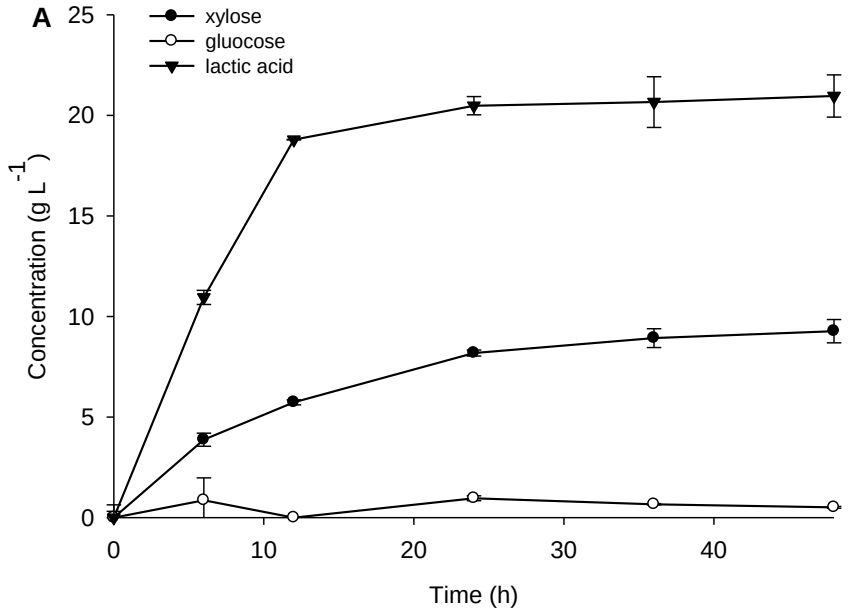
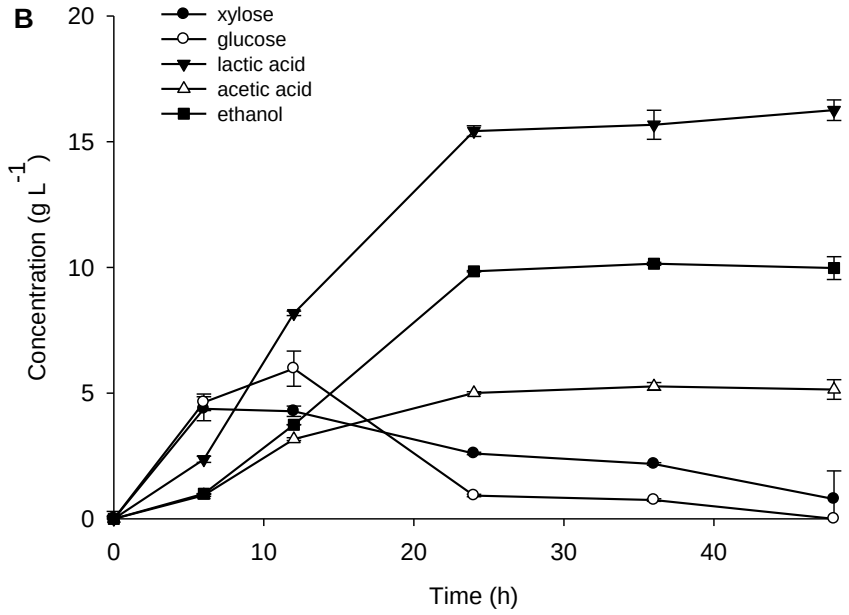


Figure 4.3 Lactic acid production from poplar hydrolysate by sequential fermentation


A
xylose
gluocose
lactic acid
Concentration (g L-1)
Time (h)
0
5
10
15
20
25
0
10
20
30
40


B
xylose
glucose
lactic acid
acetic acid
ethanol
Concentration (g L-1)
Time (h)
0
5
10
15
20
0
10
20
30
40

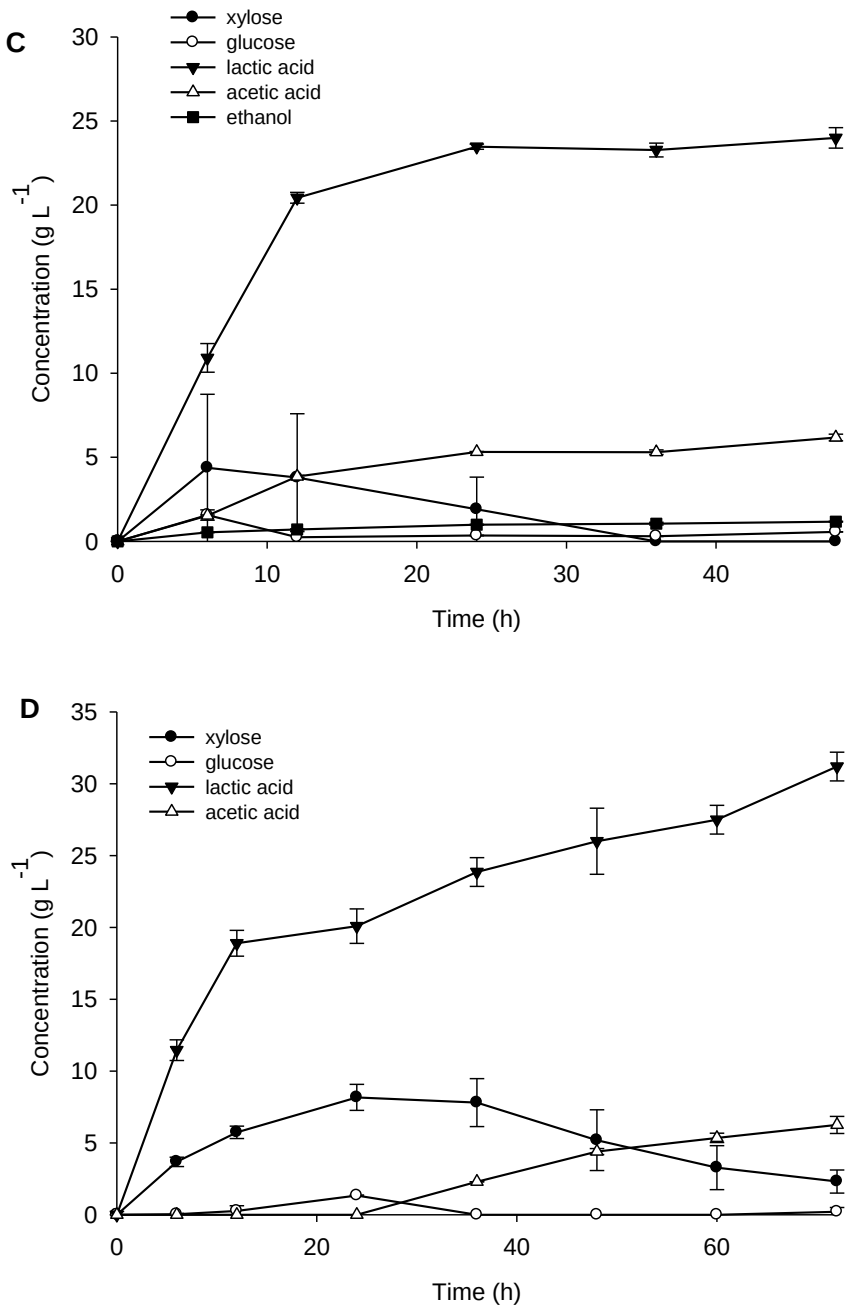


Figure 4.4 Lactic acid production from pretreated corn stover via SSF process by (A) *L. plantarum*; (B) *L. brevis*; (C) simultaneous cultivation of *L. plantarum* and *L. brevis*; (D) sequential cultivation of *L. plantarum* and *L. brevis*

Chapter 5 - D-lactic acid production from renewable lignocellulosic biomass by genetically modified *Lactobacillus plantarum*

Abstract

D-lactic acid is of great interest because of the increasing market demand of bio-based poly-lactic acid (PLA). Blending poly-L-lactic acid (PLLA) with poly-D-lactic acid (PDLA) greatly improves PLA's mechanical and physical properties. Corn stover and sorghum stalks treated with 1 % sodium hydroxide were investigated as possible substrate for D-lactic acid production by both sequential saccharification and fermentation (SHF), and simultaneous saccharification and fermentation (SSF). A commercial cellulase (Cellic CTec2) was used for hydrolysis of lignocellulosic biomass, and L-lactate deficient mutant strain *Lactobacillus plantarum* NCIMB 8826 Δ*ldhL1* and its derivative harboring a plasmid (Δ*ldhL1*-pCU-P*xylAB*) used for xylose assimilation were used for fermentation. The SSF process demonstrated the advantage of avoiding feedback inhibition of released sugars from lignocellulosic biomass, thus significantly improving D-lactic acid yield and productivity. The highest D-lactic acid concentration (27.0 g L^{-1}) and productivity (0.75 g L^{-1} h^{-1}) was obtained from corn stover using Δ*ldhL1*-pCU-P*xylAB* via SSF process. The recombinant strain produced higher concentration of D-lactic acid than mutant strain by using the xylose present in lignocellulosic biomass. A small amount of acetic acid was also produced from pentose sugars (xylose and arabinose) via the phosphoketolase pathway. Our findings demonstrated the potential of using metabolically engineered lactic acid bacteria in a cost-effective system to produce D-lactic acid.

Introduction

Demand for lactic acid is increasing because of its wide applications in the food, cosmetic and packaging industries. Annual demand for lactic acid is estimated to be roughly 130 to 150 kilotons per year (Wee 2006). Lactic acid can be produced either by chemical synthesis or fermentation. One advantage of microbial fermentation is that certain microorganism can produce optically pure D (+) or L (-) lactic acid (John et al. 2007). The optical purity of lactic acid is crucial to the physical properties of poly-lactic acid (PLA), which is a biodegradable plastic that can be produced from inexpensive, renewable and abundantly available biomass resources (Okano et al. 2009c). A stereocomplex PLA, which is composed both poly-L-lactic

acid (PLLA) and poly-D-lactic acid (PDLA), is also of great interest due to its superior thermostability (Okano et al. 2010).

Lactic acid is a commodity chemical that can be produced in large quantities at a relatively low price. Feasible economic production of lactic acid requires inexpensive raw materials such as agriculture residues and forestry sources (Nguyen et al. 2013). Agricultural residues such as corn stover, sorghum stalks are rich in carbohydrates but low in protein and are difficult to digest, which limits their utilization as livestock feed or human food (John et al. 2007). Corn stover including the leaves, husk and stalks, comprises up to half of the crop's yield is one of the most abundant agriculture residues in United States (Li et al. 2004). Raw corn stover consists about 49.6 % glucan, 25.1 % xylan and 23.7 % of lignin (Guragain et al. 2013), and it has been studied for its potential contribution to the production of biofuels (Zambare et al. 2012) and biobased lactic acid (Cui et al. 2011; Miura et al. 2004; Zhang and Vadlani 2013). Sorghum has been recommended as a feedstock for biofuels production, and of potential crops, has the highest water use efficiency and higher tolerance to low soil fertility (Xin et al. 2009). Genetic screening through chemical mutagenesis of sorghum generated several useful traits. For example, the brown midrib mutant that showed lower lignin content and higher enzymatic conversion efficiency (Cotton et al. 2013). Most studies were focused on conversion of sweet sorghum juice into lactic acid (Richter and Träger 1994), however, production of D-lactic acid from structural carbohydrates in sorghum has not been widely investigated.

The carbohydrate present in the lignocellulosic biomass must be hydrolyzed into fermentative sugars for digestion by microorganisms. The enzymatic hydrolysis method is most widely used for lignocellulosic biomass, but one well-known disadvantage of enzymatic catalysis is feedback inhibition by sugars released from the biomass (Olofsson et al. 2008). Thus, simultaneous saccharification and fermentation (SSF) is usually used to prevent enzyme inhibition, which combines enzymatic hydrolysis and fermentation into a single step and reduces reactor volume and increase productivity (Olofsson et al 2008).

Lactobacillus and *Lactococcus* species are the most studied groups for lactic acid production (Li and Cui 2010). Lactic acid bacteria (LAB) can be classified into three groups: homofermentative, heterofermentative and facultative heterofermentative. Homofermentative LAB can convert sugars exclusively into lactic acid through Embden-Meyerhof pathway (EMP), whereas heterofermentative LAB metabolize sugars through the phospoketolase (PK) pathway to

produce lactic acid along with other by-products such as acetic acid, ethanol, and/or carbon dioxide (Hofvendahl and Hahn-Hagerdal 2000). The third group, facultative heterofermentative LAB, (*Lactobacillus plantarum*, for example), metabolizes hexose sugars through EMP pathway, but uses pentose sugars through the PK pathway (Kleerebezem et al. 2003). *Lactobacillus plantarum* is an industrially important strain which can use a wide range of carbohydrates including glucose, fructose, arabinose (Okano 2009a, 2009c), but it is not able to use xylose, which is a dominant sugar from hemicellulose. The genomes of several *L. plantarum* strains have been sequenced (Kleerebezem et al. 2003; Li et al. 2013), and several gene manipulation methods have been developed for *L. plantarum* (Rud et al. 2006). Okano et al (2009c) deleted the L-lactate dehydrogenase gene of *L. plantarum* NCIMB 8826, and the mutant strain *L. plantarum* NCIMB8826 Δ*ldhL1* successfully produce D-lactic acid with optical purity of 99.7 % from pure glucose. Xylose assimilation plasmid pCU-P*xylAB* was introduced into this L-lactate dehydrogenase-deficient strain and the resulting recombinant strain was able to produce D-lactic acid from xylose with a yield of 0.57 g g^{-1} and 99.9 % optical purity (Okano et al. 2009b) These engineered strains perform well in the fermentation of pure sugars, but their performance on real biomass sugars has not been reported. Therefore, the objective of this study was to evaluate lactic acid production ability of mutant and recombinant *L. plantarum* from lignocellulosic biomass.

Materials and methods

Microorganisms and culture conditions

Table 5.1 lists the microorganisms and plasmid used in this study. Pre-culture were prepared by growing *L. plantarum* NCIMB 8826 Δ*ldhL1* in MRS broth (OXOID Ltd. Basingstoke, Hampshire, England) and *L. plantarum* NCIMB 8826 Δ*ldhL1*-pCU-P*xylAB* in MRS broth with 25 μg mL^{-1} of erythromycin. All cultures were grown in a temperature controlled shaker (Innova 4300, New Brunswick Scientific, NJ) at 37 ℃ until OD_{600} reached around 5.0. Pre-culture were used to inoculate fermentation media at 5 % (v/v).

Lignocellulosic biomass composition analysis and pretreatment

Corn stover was obtained from the Kansas State University Agronomy Farm in Manhattan and Tribune, Kansas. Sorghum stalks was obtained from Taxes A&M University and

ground by Mesa Associate Inc. The biomass samples were obtained from universities-based field studies and required no special permission and did not involve endangered or protected species. Both corn stover and sorghum stalks were treated with 1 % NaOH (w/v) using the method described by Guragain et al. (2013). The composition of sorghum stalks was determined by following the protocol NREL/TP-510-42618 (Sluiter et al. 2008).

Enzymatic hydrolysis of corn stover and sorghum stalks

Enzymatic hydrolysis of corn stover and sorghum stalks was done using the method modified from Zhang and Vadlani (2013). Two g of alkali-treated corn stover and sorghum stalks were suspended in 40 mL of 50 mM citrate buffer (pH 5), and Cellic CTec2 obtained from Novozymes Inc. (Franklinton, NC) was added at dosage of 8 FPU per gram of lignocellulosic biomass. Biomass hydrolysis was performed at 50℃ with agitation of 150 rpm. Samples (1 mL) were withdraw from reaction media at 0, 12, 24 and 48 h, centrifuged, filtered and analyzed by HPLC. Sugar yield was reported by the amount of released sugars divided by the amount of biomass.

Fermentation experiments

The effect of initial glucose concentration was tested on D-lactic acid production for mutant strain Δ*ldhL1* in 100 mL Wheaton serum bottles (Fisher Scientific, Pittsburgh, PA) containing 50 mL modified MRS broth consisting of different concentrations of glucose (30 g L^{-1} , 60 g L^{-1} , and 90 g L^{-1}), supplemented with 10 g L^{-1} of peptone, 5 g L^{-1} of yeast extract, 2 g L^{-1} of ammonium citrate, 2 g L^{-1} of K_2HPO_4, 0.1 g L^{-1} of $MgSO_4.7H_2O$, 0.05 g L^{-1} of $MnSO_4.4H_2O$.

An experiment with a mixture of glucose and xylose for recombinant strain Δ*ldhL1*-pCU-P*xylAB* was also performed in 100-mL Wheaton serum bottles containing a mixture of 60 g L^{-1} glucose and 40 g L^{-1} xylose supplemented with 10 g L^{-1} of peptone, 5 g L^{-1} of yeast extract, 2 g L^{-1} of ammonium citrate, 2 g L^{-1} of K_2HPO_4, 0.1 g L^{-1} of $MgSO_4.7H_2O$, and 0.05 g L^{-1} of $MnSO_4.4H_2O$, and erythromycin was added at final concentration of 25 μg mL^{-1}. The initial pH of the media was adjusted to 6.5 using 10 N sodium hydroxide, and 3 % (w/v) calcium carbonate was added to maintain the pH during fermentation. All serum bottles were incubated at 37˚C with agitation of 150 rpm.

Fed-batch xylose fermentation of recombinant strain Δ*ldhL1*-pCU-P*xylAB* was performed in a 7-L fermenter with 5-L working volume (Bioflo 110, New Brunswick Scientific Inc.,

Enfield, CT). Fermentation medium containing 40 g L^{-1} xylose supplemented with all the other components of modified MRS medium. After 46 h fermentation, 1L of fermentation broth was pumped out and 1 L of feeding solution containing 200 g xylose was pumped in. During the fermentation, the temperature was maintained at 37 ℃ and agitation was 150 rpm. pH was maintained at 6.5 by pumping in 10 N sodium hydroxide. Erythromycin was added at final concentration of 25 µg mL^{-1}.

Sequential hydrolysis and fermentation (SHF) with corn stover and sorghum stalks were performed in 100-mL serum bottles. Two g of dried corn stover and sorghum stalks were hydrolyzed for 48 h. The hydrolysates were then centrifuged in Sorvall RC 5C plus super speed centrifuge (Beckman Coulter, Inc., Brea, CA) at 10,000 ×g for 10 min, supernatants were collected and the pH was adjusted to 6.5 using 10 N NaOH. Supernatants were autoclaved at 121℃ for 15 min and supplemented with 5 mL concentrated nutrient solution containing 100 g L^{-1} of peptone, 50 g L^{-1} of yeast extract, 20 g L^{-1} of ammonium citrate, 20 g L^{-1} of K_2HPO_4, 1 g L^{-1} of $MgSO_4.7H_2O$, and 0.5 g L^{-1} of $MnSO_4.4H_2O$, and final fermentation volume was adjusted to 50 mL by adding water. Calcium carbonate was added at 3 % (w/v) to control the pH. Mutant strain Δ*ldhL1* and recombinant Δ*ldhL1*-pCU-P*xylAB* strain were added at inoculum of 5 % (v/v). Fermentation was performed at 37℃ with 150 rpm agitation. Erythromycin was added at final concentration of 25 µg mL^{-1} when needed.

Simultaneous saccharification and fermentation (SSF) with biomass was conducted in 100-mL serum bottles. Two g of dried pretreated corn stover or sorghum stalks were suspended in 50 mL of 50 mM sodium citrate buffer (pH 5) supplemented with all the components except sugars of the modified MRS medium. Cellic CTec2 was added at 8 FPU per gram of lignocellulosic biomass. Mutant strain Δ*ldhL1* and recombinant Δ*ldhL1*-pCU-P*xylAB* strain were added at inoculum of 5 % (v/v). Temperature was maintained at 37 ºC, and the agitation rate was maintained at 150 rpm. Erythromycin was added at final concentration of 25 µg mL^{-1} when needed.

Electrotransformation procedures

pCU-P*xylAB* plasmid was propagated by transformation into *Escherichia coli* DH5α following the methods of Sambrook et al. (2001). *E.coli* transformants were selected on LB agar plates containing 250 µg mL^{-1} of erythromycin. pCU-P*xylAB* plasmids were extracted from

E.coli using IBI plasmid extraction kit (MidSci, St.Louis, MO) following the manufacturer's instruction. pCU-P*xylAB* plasmid (Okano et al. 2009b) was transformed into *L. plantarum* NCIMB 8826 Δ*ldhL1* using the method modified from Narita et al. (2006). *L. plantarum* NCIMB 8826 Δ*ldhL1* was pre-cultivated overnight in a test tube containing 5 mL of MRS broth. One mL of the overnight culture was added into 100 mL fresh MRS broth and cultivated at 37℃ until OD_{600} value reached about 0.7. Cells were washed three times with ice-cold water followed by three times of wash buffer (272 mM sucrose, 7 mM HEPES, 1mM $MgCl_2$, pH 7.4), then suspended in 1 mL 0.3 mol L^{-1} of sucrose. One μg of plasmid DNA was mixed with 100 μL of competent cells and was incubated on ice for 5 min, then electroporated using a Gene pulser Xcell electroporator (Bio-Rad, Hercules, CA) in 0.1 cm cuvettes at 1.5 kV, 25 μFD and 200 Ohm. Five hundred microliter of fresh MRS broth was immediately added, and cells were incubated at 37℃ for 2 h before plated on MRS agar with 25 μg mL^{-1} erythromycin. The plates were incubated at 37˚C for 2 to 3 days.

Analytical methods

OD_{600} of cultures were measured using a Shimadzu UV-spectrometer (UV-1650PC, Torrance, CA). Cell dry mass was determined by method described by Zhang et al. (2013). Fermentation samples were centrifuged at 15,000 × g for 10 min in a micro-centrifuge (Eppendorf, Hauppauge, NY, USA). Supernatant was acidified with 1 N HCl, and diluted 10 times with deionized water. Lactic acid, and acetic acid were qualified using Shimadzu HPLC system equipped with a refractive index (RI) detector (RID-10A), a UV/VIS detector (SPD-M20A) and Rezex ROA organic acid column (150 × 7.8 mm, Phenomenex Inc., Torrance, CA, USA). 0.005 N H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL min^{-1}. Temperatures of the column and detector were maintained at 83 and 40 °C, respectively. Sugars were measured using a phenomenex RCM monosaccharide column (300 ×7.8 mm, Phenomenex, Torrance, CA, USA) using deionized water as mobile phase at 0.6 mL min^{-1}. Lactic acid optical purity was measured using the method described by Zhang and Vadlani (2013).

SAS software version 9.4 (SAS Inc., Cary, NC, USA) was used to analyze experimental data by applying PROC GLM. Ryan-Einot-Gabriel-Welsh (REGWQ) multiple range test was performed to compare the difference among means. All difference were considered significant at α=0.05 level.

Results and discussion

Effect of initial glucose concentration

Substrate inhibition is one of the major problem associated with lactic acid production. Substrate inhibition was observed for the Δ*ldhL1* strain, for which maximum lactic acid yield (0.88 g g^{-1}) and productivity (1.11 g h^{-1} L^{-1}) were obtained when the initial glucose concentration was 60 g L^{-1}(Table 5.2). When the initial glucose increased to 90 g L^{-1}, the yield decreased to 0.69 g g^{-1} and productivity decreased to 0.86 g h^{-1} L^{-1}. Therefore initial glucose concentration was set to 60 g L^{-1} in subsequent mixture sugar fermentation experiment.

*D-lactic acid fermentation from xylose by Δ*ldhL1-pCU-PXylAB*

Transformed *L. plantarum* cells were selected on MRS agar plates with 25 µg mL^{-1} erythromycin. Cell growth with xylose was evaluated by growing cells in modified MRS medium containing 40 g L^{-1} xylose as sole carbon source. After 12 h cultivation, the OD_{600} of Δ*ldhL1*-pCU-P*xylAB* was 2.1, while the control strain Δ*ldhL1* (without plasmid) showed poor growth using xylose as the sole carbon source (OD_{600} was 0.54).

A fed-batch xylose fermentation was performed using Δ*ldhL1*-pCU-P*xylAB* (Fig. 5.1A). All xylose was consumed by the end of first-stage fermentation, and 22.3 g L^{-1} of D-lactic acid with 99.9 % optical purity was produced along with 16.4 g L^{-1} of acetic acid. Fresh xylose solution was added at 46 h, and fermentation continued for next 62 h. Δ*ldhL*1-pCU-P*xylAB*, consumed xylose much more slowly in the second stage than the first; at the end of fermentation, 4.8 g L^{-1} xylose remained, the lactic acid concentration was 37.1 g L^{-1}, and the acetic acid concentration was 28.2 g L^{-1}. Although the xylose consumption rate slowed, the lactic acid concentration had not plateau, suggesting the recombinant strain would keep producing lactic acid if more xylose were added. These results were in agreement with those reported by Okano et al. (2009b).

*D-Lactic acid fermentation from mixture of glucose and xylose by Δ*ldhL1-pCU-PXylAB*

Lignocellulosic biomass hydrolysate consists of both glucose and xylose, and efficient utilization of both sugars is crucial for economical production of D-lactic acid. Carbon catabolite repression widely exists in many lactic acid bacteria, which means glucose is always the

preferred carbon and energy source, but it represses the utilization of other sugars (Goerke et al. 2008). Δl*dhL1*-pCU-P*xylAB* did not show a hierarchical sugar utilization pattern (Fig. 5.1B). Glucose and xylose were consumed simultaneously, but glucose was consumed faster than xylose; at the end of 72 h fermentation, all glucose was consumed, but 10.1 g L^{-1} of xylose was left. Lactic acid concentration was 67.4 g L^{-1} with optical purity of 99.9 %. Acetic acid concentration was 19.7 g L^{-1}. The yield of D-lactic acid from both glucose and xylose was 0.75 g g^{-1} and productivity was 0.94 g L^{-1} h^{-1}. These results were comparable with results reported by Yoshida et al. (2011). In our previous study, the heterofermentative strain *Lactobacillus brevis* used xylose simultaneously with glucose, which gave a lactic acid yield of 0.52 g g^{-1} from mixture of glucose and xylose (Zhang and Vadlani 2015). Δ*ldhL1*-pCU-P*xylAB* is a facultative heterofermentative strain that uses glucose through the EMP and xylose through the PK pathway (Okano et al. 2009c), which gives better lactic acid yield then heterofermentative lactic acid bacteria.

Enzymatic hydrolysis of corn stover and sorghum stalks

After alkali pretreatment, corn stover consisted of around 53.9 % (w/w) glucan, 29.7 % (w/w) xylan, less than 5 % arabinan (Guragain et al. 2013; Huang et al. 2009). The composition of alkali treated sorghum stalks was 44.4 % (w/w) glucan, 24.3 % (w/w) xylan, and 2.0 % (w/w) arabinan. Therefore, 1 g pretreated corn stover would yield 0.60 g of glucose and 0.34 g of xylose, and 1 g of pretreated sorghum stalks would yield 0.49 g of glucose and 0.28 g of xylose calculated by the equation: glucose (g) = glucan (g)/0.9; xylose (g) = xylan (g)/0.88 (Sluiter et al. 2008). Corn stvoer and sorghum stalks in this study showed a similar enzymatic hydrolysis profile (Fig. 5.2). Glucose yield was not significantly increased after 24 h for either corn stover or sorghum stalks. Xylose yield increased 8 % and 15 % from 24 h to 48 h for corn stover and sorghum stalks, respectively. At the end of 48 h of corn stover hydrolysis, glucose yield was 0.52 g per g of biomass, which was 86.7 % of theoretical yield, and xylose yield was 0.22 g per g of biomass, which was and 64.7 % of theoretical yield. At the end of 48 h of sorghum stalks hydrolysis, glucose yield was 0.43 g per g of biomass, which was 87.8 % of theoretical yield, and xylose yield was 0.22 g per g of biomass, which was 78.6 % of theoretical yield. Therefore, the 48-h biomass hydrolysate were used for the following SHF experiments.

D-lactic acid production from corn stover and sorghum stalks via SHF

Sequential hydrolysis and fermentation experiments were conducted as described in Materials and Methods. Δ*ldhL1* consumed all the glucose (17.7 g L^{-1}) and arabinose (1.0 g L^{-1}) within 12 h; at the end of 12 h of fermentation, 15.9 g L^{-1} of D-lactic acid with optical purity of 99.6 % was produced (Table 5.3). The yield of D-lactic acid from glucose and arabinose was 0.85 g g^{-1}, which was slightly higher than the yield (0.83 g g^{-1}) from a homofermentative *Lactobacillus delbrueckii* ATCC 9649 in our previous study (Zhang and Vadlani 2013). Δ*ldhL1*-pCU-P*xylAB* consumed all of the glucose (17.6 g L^{-1}), xylose (7.5 g L^{-1}) and arabinose (1.0 g L^{-1}) from corn stover hydrolysate within 24 h and produced 19.7 g L^{-1} of D-lactic acid along with 4.2 g L^{-1} of acetic acid (Table 5.3). For sorghum stalks hydrolysate, Δ*ldhL1* consumed all of the glucose (14.2 g L^{-1}) and arabinose (0.74 g L^{-1}) within 12 h and produced 12.5 g L^{-1} of D-lactic acid with a yield of 0.84 g g^{-1}. Δ*ldhL1*-pCU-P*xylAB* consumed all sugars (14.0 g L^{-1} of glucose, 7.0 g L^{-1} of xylose, and 0.74 g L^{-1} of arabinose) within 24 h and produced 15.6 g L^{-1} of D-lactic acid along with 5.0 g L^{-1} acetic acid (Table 5.3). A small amount of acetic acid was detected when using Δ*ldhL1* to produce D-lactic acid from corn stover and sorghum stalks in SHF. Δ*ldhL1* was reported as capable of using arabinose to produce lactic acid and acetic acid (0.44 g lactic acid and 0.32 g acetic acid produced per g arabinose consumed, respectively) (Okano et al. 2009a), but acetic acid yield from corn stover and sorghum stalks hydrolysate by mutant strain Δ*ldhL1* in our study exceeded the theoretical yield of acetic acid produced from arabinose (0.6 g g^{-1}). This is probably due to the loosely controlled anaerobic fermentation environment, which allowed small amount of oxygen in the bottles; thereby the metabolic pattern of glucose shifted and resulted in additional production of acetic acid along with lactic acid (Litchfield 1996).

D-Lactic acid production from corn stover and sorghum stalks via SSF

SSF process has several advantages compared to SHF, the most important reason for using SSF is to avoid end-product inhibition in the hydrolysis (Olofsson et al. 2008). Sugars released during hydrolysis are removed by *in situ* fermentation, which reduces the feedback inhibition to the cellulolytic enzymes and may improve the yield and productivity of lactic acid. The optimal temperature range for CTec2 is 45 to 50℃, but Δ*ldhL1* and Δ*ldhL1*-pCU-P*xylAB* grew poorly above 45℃ (OD_{600} ~ 0.2), so the temperature was set at 37℃. Xylose accumulated up to 6.8 g L^{-1} during the fermentation using corn stover, but glucose was barely detected during

the fermentation, which indicated that the glucose released from hydrolysis was rapidly consumed by Δ*ldhL1* (Fig. 5.3A). D-lactic acid concentration increased rapidly during the first 12 h then slowed down, at the end of fermentation, D-lactic acid concentration was 21.1g L^{-1} with optical purity of 99.5%. Fig. 5.3B shows the SSF profile of Δ*ldhL1*-pCU-P*xylAB* using corn stover. Unlike Δ*ldhL1*, glucose and xylose concentration were accumulated to the maximum level of 1.8 and 1.2 g L^{-1} at 6 h, respectively, after 6 h, the concentration of both glucose and xylose were maintained below 1 g L^{-1} during remaining fermentation time, and 27.0 g L^{-1} of D-lactic acid was produced along with 9.2 g L^{-1} of acetic acid (Table 5.4). Because of xylose utilization ability, Δ*ldhL1*-pCU-P*xylAB* gave significantly higher D-lactic acid concentration, yield (0.67 g g^{-1}) and productivity (0.75 g L^{-1} h^{-1}) compared with Δ*ldhL1* (yield of 0.53 g L^{-1}, productivity of 0.58 g L^{-1} h^{-1}) in SSF with corn stover. Δ*ldhL1* showed an overall yield similar to that of *L. delbrueckii*, which was used to produce D-lactic acid from corn stover via the SSF process (Zhang and Vadlani 2013). Δ*ldhL1*-pCU-P*xylAB* yielded similar lactic acid concentration (27.0 g L^{-1}) from corn stover compared with a sequential co-fermentation system that produced 31.2 g L^{-1} of lactic acid from corn stover via SSF in our previous study (Zhang and Vadlani 2015), but lactic acid productivity of Δ*ldhL1*-pCU-P*xylAB* was greatly improved.

Fig. 5.3C shows the SSF profile of Δ*ldhL1* of using sorghum stalks as substrate. Glucose reached to the maximum concentration of 1.2 at 5 h then decreased to 0 g L^{-1} in the next 5 h. Xylose concentration increased to 6.7 g L^{-1} at 34 h. D-lactic acid concentration was 15.7 g L^{-1} with optical purity of 99.4% and acetic acid was 3.7 g L^{-1} (Table 5.4). Fig. 5.3D shows the SSF profile of Δ*ldhL1*-pCU-P*xylAB*; similar to corn stover, glucose concentration reached a maimum of 1.2 g L^{-1} and xylose concentrations reached a maximum 1.4 g L^{-1} at 5 h. Glucose concentration remained below 1 g L^{-1} during the remaining SSF time and xylose concentration was maintained at close to 1 g L^{-1}. D-Lactic acid (21.6 g L^{-1}) with optical purity of 99.0 % was produced along with 7.8 g L^{-1} of acetic acid. The D-lactic acid yield of Δ*ldhL1* from sorghum stalks was 0.39 g g^{-1} and productivity was 0.46 g L^{-1} h^{-1}. D-lactic acid yield of Δ*ldhL1*-pCU-P*xylAB* from sorghum stalks was 0.54 g g^{-1}, and productivity was 0.64 g L^{-1} h^{-1} (Table 5.4).

In our study, SSF with SHF were comparatively evaluated; lactic acid concentration, overall yield, and productivity were greatly improved. Other advantages of using SSF include immediate utilization of sugars, thereby avoiding any substrate inhibition. In the SSF, the hydrolysis and fermentation occur in different reactors. Separation of sugars from biomass

residues in the hydrolysates and then transferring the sugar solution into fermentation reactor result in sugar loss. By contrast, in the SSF process, hydrolysis and fermentation are performed in the same reactor, which avoids the potential sugar loss during the sugar transfer, and also decreases the capital investment because less vessels are needed in the SSF process. Therefore, the overall yield and productivity of D-lactic acid obtained in SSF process were greatly increased compared to that obtained in SHF process.

Conclusions

Both mutant and recombinant strains were capable of producing D-lactic acid at high yield and optical purity from pure sugars. Δ*ldhL1*-pCU-P*xylAB* used xylose to produce high yields of D-lactic acid and was able to use xylose simultaneously with glucose, which is an important advantage when using lignocellulosic biomass as substrate to produce lactic acid. D-lactic acid production was successfully demonstrated from corn stover and sorghum stalks. Δ*ldhL1*-pCU-P*xylAB* was able to produce 20 % more D-lactic acid than Δ*ldhL1* from lignocellulosic biomass. Overall yield increased about 38 % and productivity almost three fold when the SSF process was applied to produce D-lactic acid. To our knowledge this is the first report that successfully demonstrates D-lactic acid production from lignocellulosic biomass using specifically designed genetically engineered lactic acid bacteria. Δ*ldhL1*-pCU-P*xylAB* has vast potential in the industrial production of D-lactic acid.

Table 5.1 Bacterial strains and plasmid

Strains and plasmid	Relevant characteristics	Antibiotic resistance	Reference or source
Escherichia coli			
DH5α	lacZ ΔM15, recA1, endA1		Invitrogen
Lactobacillus plantarum			
Δ*ldhL1*	*L.plantarum* NCIMB 8826 L-lactate dehydrogenase gene1 deletion		Okano et al. 2009c
Δ*ldhL1*-pCU-P*xylAB*	*L.plantarum* NCIMB 8826ΔldhL1 harboring xylose assimilation plasmid	Erythromycin	Okano et al. 2009b
Plasmid			
pCU-P*xylAB*	Expression vector containing the *xylAB* operon under the control of *clpC* UTLS promoter	Erythromycin	Okano et al. 2009b

Table 5.2 D-lactic acid production from different initial glucose concentration

Initial glucose ($g\ L^{-1}$)	D-lactic acid ($g\ L^{-1}$)	Productivity[1] ($g\ L^{-1}\ h^{-1}$)	Yield [2] ($g\ g^{-1}$)
30	24.6 ± 0.3^{C}	1.03 ± 0.01^{B}	0.82 ± 0.01^{B}
60	53.1 ± 0.7^{B}	1.11 ± 0.01^{A}	0.89 ± 0.01^{A}
90	62.2 ± 0.9^{A}	0.86 ± 0.01^{C}	0.69 ± 0.01^{C}

Data represent mean values and standard errors based on three replications ($p < 0.05$). Values with different letters in the same column are significantly different.

[1] Productivity was defined as the amount of D-lactic acid produced per liter per hour. In SHF, the 48 h hydrolysis time was included in productivity calculation.

[2]Yield was calculated by dividing the amount of D-lactic acid by the amount of sugar consumed.

Table 5.3 D-lactic acid production from alkali-treated biomass via SHF

Strain name	D-lactic acid ($g\ L^{-1}$)	Acetic acid ($g\ L^{-1}$)	Yield [1] ($g\ g^{-1}$)	Overall yield [2] ($g\ g^{-1}$)	Productivity[3] ($g\ L^{-1}\ h^{-1}$)	Optical purity [4] (%)
Corn stover						
Δ*ldhL1*	15.9 ± 0.2^{B}	1.3 ± 0.3^{C}	0.85 ± 0.01^{A}	0.40 ± 0.01^{B}	0.26 ± 0.01^{A}	99.6
Δ*ldhL1*-pCU-P*xylAB*	19.7 ± 0.6^{A}	4.2 ± 0.1^{B}	0.75 ± 0.01^{B}	0.49 ± 0.01^{A}	0.27 ± 0.01^{A}	99.3
Sorghum stalks						
Δ*ldhL1*	12.5 ± 0.7^{C}	1.5 ± 0.3^{C}	0.84 ± 0.05^{A}	0.31 ± 0.02^{C}	0.21 ± 0.01^{B}	99.2
Δ*ldhL1*-pCU-P*xylAB*	15.6 ± 0.5^{B}	5.0 ± 0.1^{A}	0.72 ± 0.04^{B}	0.39 ± 0.01^{B}	0.22 ± 0.01^{B}	99.4

Data represent mean values and standard errors based on three replications ($p < 0.05$). Values with different letters in the same column are significantly different.

[1]Yield was calculated by dividing the amount of D-lactic acid by the amount of sugar consumed

[2]Overall yield was calculated by dividing the amount of D-lactic acid by the amount of biomass used.

[3] Productivity was defined as the amount of D-lactic acid produced per liter per hour. In SHF, the 48 h hydrolysis time was included in productivity calculation.

[4] Optical purity (OP) calculated based on the equation: OP=100×(D-lactic acid concentration)/(Total lactic acid concentration)

Table 5.4 D-lactic acid production from alkali-treated biomass via SSF

Strain name	D-lactic acid ($g\ L^{-1}$)	Acetic acid ($g\ L^{-1}$)	Overall yield [1] ($g\ g^{-1}$)	Productivity[2] ($g\ L^{-1}\ h^{-1}$)	Optical purity [3] (%)
Corn stover					
Δ*ldhL1*	21.1 ± 0.3^{B}	2.6 ± 0.1^{D}	0.53 ± 0.01^{B}	0.58 ± 0.01^{C}	99.5
Δ*ldhL1*-pCU-P*xylAB*	27.0 ± 0.3^{A}	9.2 ± 0.4^{A}	0.67 ± 0.01^{A}	0.75 ± 0.01^{A}	99.5
Sorghum stalks					
Δ*ldhL1*	15.7 ± 0.2^{C}	3.7 ± 0.1^{C}	0.39 ± 0.01^{C}	0.46 ± 0.01^{D}	99.4
Δ*ldhL1*-pCU-P*xylAB*	21.6 ± 0.3^{B}	7.8 ± 0.1^{B}	0.54 ± 0.01^{B}	0.64 ± 0.01^{B}	99.0

Data represent mean values and standard errors based on three replications ($p < 0.05$). Values with different letters in the same column are significantly different.

[1]Overall yield was calculated by dividing the amount of D-lactic acid by the amount of biomass used.

[2] Productivity was defined as the amount of D-lactic acid produced per liter per hour.

[3] Optical purity (OP) calculated based on the equation: OP=100×(D-lactic acid concentration)/(Total lactic acid concentration)

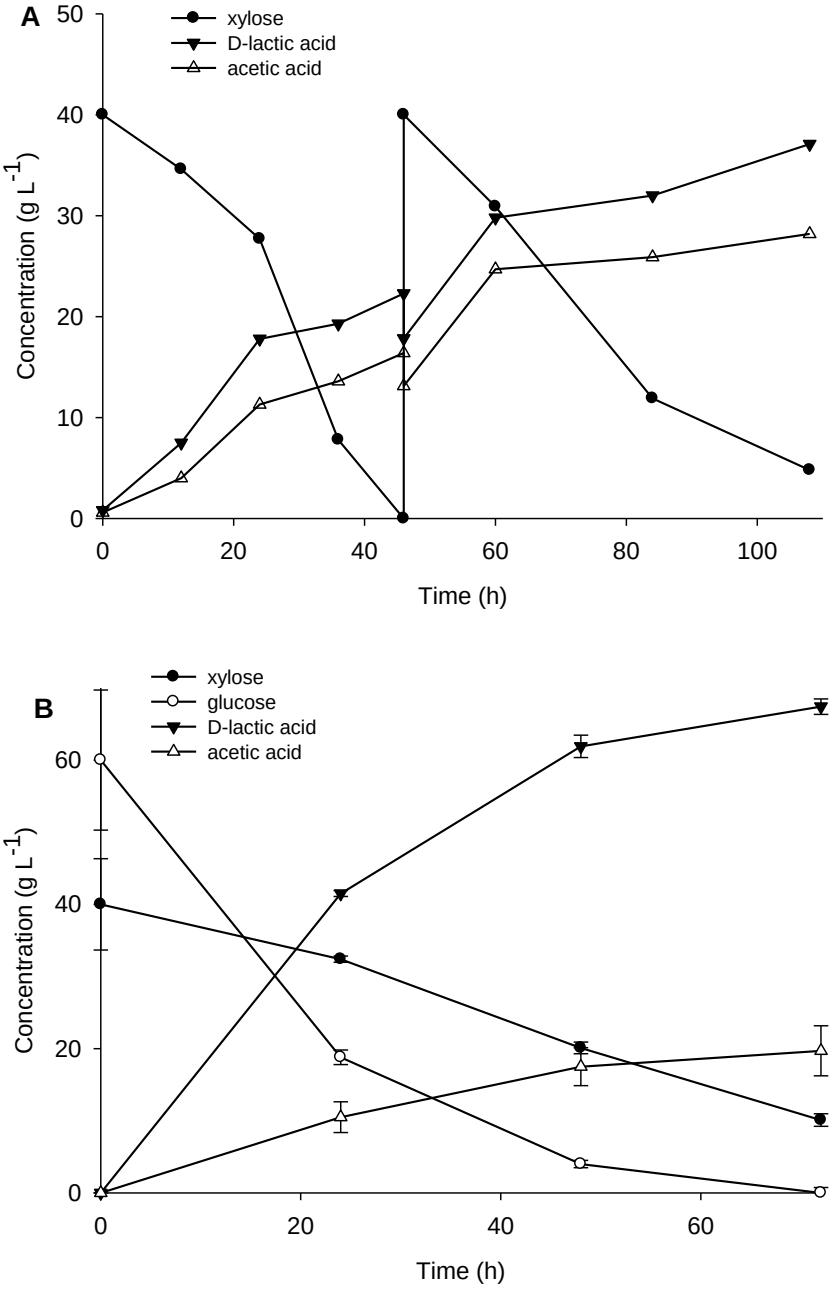


Figure 5.1 D-lactic acid production from refined sugars: (A) D-lactic acid fermentation from xylose in a 7-L fermenter; (B) D-lactic acid fermentation from mixed glucose and xylose by Δ*ldhL1*-pCU-P*xylAB*

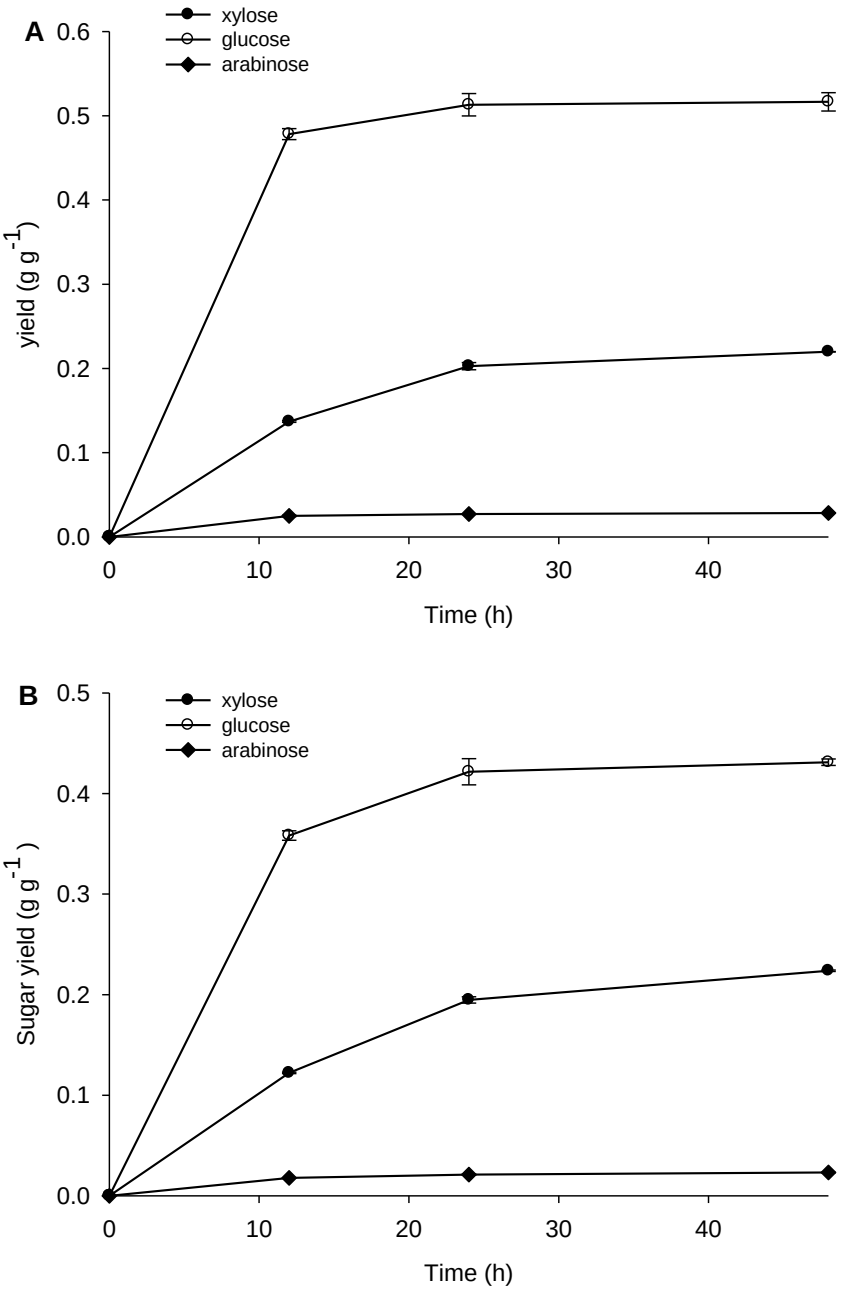
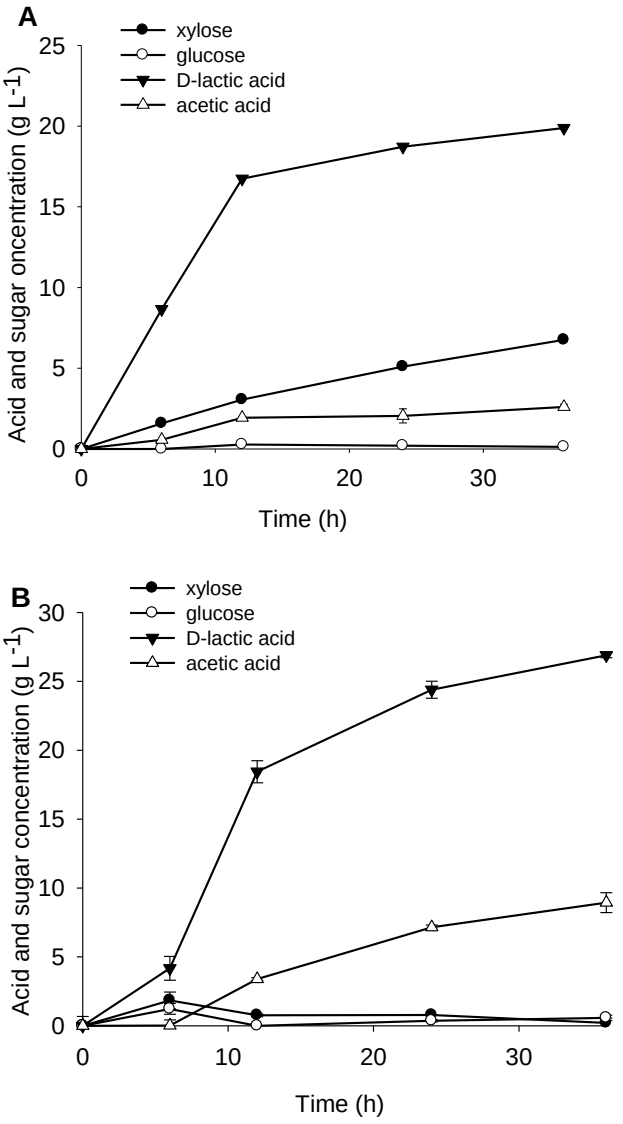


Figure 5.2 Enzymatic hydrolysis profile of alkali-treated lignocellulosic biomass: (A) Sugar yield from corn stover; (B) Sugar yield from sorghum stalks


A
xylose
glucose
D-lactic acid
acetic acid
Acid and sugar oncentration (g L-1)
Time (h)
B
xylose
glucose
D-lactic acid
acetic acid
Acid and sugar concentration (g L-1)
Time (h)

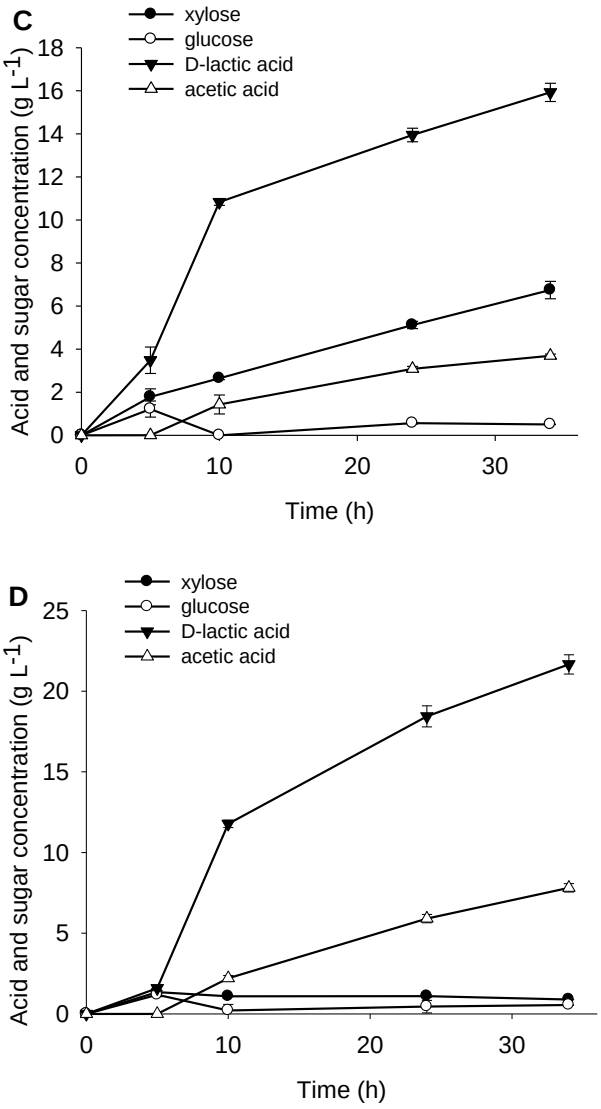


Figure 5.3 SSF profile of different lignocellulosic biomass: (A) alkali-treated corn stover using Δ*ldhL1*; (B) alkali-treated corn stover using Δ*ldhL1*-pCU-P*xylAB*; (C) alkali-treated sorghum stalks using Δ*ldhL1*; (D) alkali-treated sorghum stalks using Δ*ldhL1*-ΔpCU-P*xylAB*.

Chapter 6 - Enhanced D-lactic acid production from corn stover and soybean meal using engineered *Lactobacillus plantarum*

Abstract

D-lactic acid is used as a monomer in production of poly-D-lactic acid (PDLA), which is used to form heat-resistant stereocomplex poly-lactic acid. To produce cost-effective D-lactic acid using all sugars derived from biomass efficiently, xylose-assimilating genes encoding xylose isomerase and xylulokinase were cloned into an L-lactate deficient strain, *Lactobacillus plantarum*. The resulting recombinant strain namely *L. plantarum* NCIMB 8826 Δ*ldhL1*-pLEM415-*xylAB,* was able to produce D-lactic acid (at optical purity > 99 %) from xylose at a yield of 0.53 g g^{-1}. Simultaneous utilization of glucose and xylose to produce D-lactic acid was also achieved by this strain, and 47.2 g L^{-1} of D-lactic acid was produced from 37.5 g L^{-1} glucose and 19.7 g L^{-1} xylose. Corn stover and soybean meal extract (SBME) were evaluated as cost-effective medium components for D-lactic acid production. Optimization of medium composition using response surface methodology, resulted in enzyme loading reduced by 30 % and peptone concentration by 70 %. In addition, we successfully demonstrated D-lactic acid fermentation from corn stover and SBME in a fed-batch fermentation, which yielded 61.4 g L^{-1} D-lactic acid with an overall yield of 0.77 g g^{-1}. All these approaches are geared to attaining high D-lactic acid production from biomass sugars to produce low-cost, high-thermostable biodegradable plastics

Introduction

Optically pure lactic acid has received intensive attention because it can be used as a monomer for production of biodegradable poly-lactic acid (PLA), which is an attractive alternative to petroleum-based polymers. Physical and mechanical properties of PLA depend on the ratio of poly-D-lactic acid (PDLA) and poly-L-lactic acid (PLLA) in the copolymer. Co-crystallization occurs when PLLA is mixed with PDLA, resulting in a new stereocomplex PLA with higher melting point (Okano et al. 2010). This finding has led to increased interest in production of optically pure D-lactic acid; the commercial market of lactic acid historically has been dominated by L-lactic acid.

Lactic acid bacteria (LAB) are gram positive microorganisms that exist within plants, meat, and dairy products that produce lactic acid with high yield and productivity. LAB are either homofermentative or heterofermentative based on their end products. Homofermentative LAB such as *L. delbrueckii* (Zhang and Vadlani 2013) produce lactic acid as major end-product through the Embden-Meyerhof pathway (EMP), and are preferable for commercial-scale lactic acid production (Abdel-Rahman et al. 2013). Most homofermentative LAB cannot use pentose sugars, the dominant sugars of hemicellulose, which leads to low efficiency of using biomass sugars. On the other hand, heterofermentative LAB such as *L. brevis* (Guo et al. 2014) and *L. pentosus* (Bustos et al. 2005) use the phosphoketolase (PK) pathway, which cleaves pentose sugars to glyceraldehyde 3- phosphate (GAP) and acetyl phosphate followed by converting GAP into lactic acid. Xylose fermentation in these heterofermenters involves isomerization of xylose to xylulose and phosphorylation of xylulose to xylulose-5-phosphate (Lockman et al. 1997); however other by-products such as acetic acid, ethanol, and/or formic acid result in low lactic acid yield and additional cost in the purification step were also produced. *L. plantarum* is a facultative heterofermentative strain that ferments hexose sugars through EMP, but it also has an inducible PK pathway; arabinose was converted to xylulose-5-phosphate and further converted to lactic acid and acetic acid through the PK pathway in *L. plantarum* (Helanto et al. 2007). The goal of utilizing all major biomass sugars can be achieved during production of lactic acid if xylose-assimilating genes are introduced into *L. plantarum* and convert xylose into xylulose-5-phosphate (X5P), an intermediate in the PK pathway. Introduction of DNA into *Lactobacilli* is challenging, mainly because of the unavailability of suitable cloning vectors and efficient transformation systems (Posno et al. 1991a). The ability of plasmid to replicate itself and express foreign genes is usually unpredictable, which also add difficulty to appling recombinant DNA technology to *Lactobacillus* strains (Serror et al. 2002). pLEM415 plasmid derived from pLEM3 which was isolated from *L. fermentum* has been used to express heterologous genes in different *Lactobacillus* strains (Fons et al. 1997; Rochat et al. 2006). A well-defined constitutive promoter is preferred to an inducible promoter because inducible systems are not always easy to manage under industrial conditions (Ahmed 2006). A constitutive *clpC* promoter from *L. fermentum* BR11 displayed high activity and it was useful for starting heterologous gene expression in *Lactobacilli* strains (McCracken et al, 2000; Okano et al. 2009a; Okano et al 2009b).

Lactic acid bacteria are fastidious microorganisms; they require a wide range of growth factors including amino acids, vitamins, and fatty acids (Yadav et al. 2011). Complex nitrogen source are usually used to growth lactic acid bacteria, and yeast extract (YE) is the most effective for both microbial growth and lactic acid production (Kwon et al. 2000); however, YE is not cost-effective for commodity chemicals. The cost of YE is estimated to contribute as much as 30 % of the total production cost of lactic acid (Li et al. 2006). Soybean meal is a major residue of soybean oil extraction, and it contains 44 % crude protein and all essential amino acids including high levels of glutamic acid, a strong lactic acid bacteria growth promotant (Batal et al. 2000; Maxwell et al. 1942).

In this study, we constructed a recombinant plasmid for xylose assimilation and introduced it into *Lactobacillus plantarum* NCIMB 8826 Δ*ldhL1*. D-lactic acid production of this strain was investigated using corn stover and soybean meal extract (SBME) as substrates. The composition of the fermentation medium was optimized by response surface methodology and optimal conditions were used to produce D-lactic acid in a fed-batch fermentation.

Materials and methods

Bacterial strains and plasmids

Lactobacillus brevis ATCC 367 was purchased from the American Type Culture Collection (Manassas, VA, USA). *Lactobacillus plantarum* NCIMB 8826 Δ*ldhL1* and pCU-P*xylAB* plasmid containing the *clpC* promoter were donated by Kondo et al. (Okano et al. 2009a; 2009b), and pLEM415 plasmid was donated by Serror et al. (2002). *L. plantarum* NCIMB 8826 Δ*ldhL1* and *L. brevis* ATCC 367 were grown in MRS broth at 37 and 30℃, respectively (OXOID Ltd. Basingstoke, Hampshire, England). *Escherichia coli* DH5α was used to manipulate pLEM415-based DNA, which was grown in Luria-Bertani (LB) medium at 37 ℃. Antibiotics were added when necessary: 100 μg mL^{-1} ampicillin for *E.coli* and 25 μg mL^{-1} erythromycin for *Lactobacillus plantarum*. Table 6.1 shows the microorganisms, plasmids and primers used in this study.

Feedstock preparation

Corn stover was obtained from the Kansas State University Agronomy Farm in Manhattan and Tribune, Kansas, which was pretreated with 1 % (v/v) sodium hydroxide

according to the method described by Guragain et al. (2013). Soybean meal was obtained from the O.H. Kruse Feed Technology Innovation Center in Manhattan, Kansas. Soybean meal extract was prepared by using method modified from Zhang et al (2013). Soybean meal (60 g) was mixed with 600 mL water and shaken at room temperature with an agitation rate of 150 rpm (Innova 2350, New Brunswick Scientific, CT, USA) for 1 h. The soybean meal slurry was then centrifuged at 10,000 ×g for 10 min (Sorval RC 5C Plus, GMI Inc., MN, USA). The supernatant was collected and used as SBME for lactic acid production.

Construction of recombinant pLEM415-xylAB plasmid

The genomic DNA of *L. brevis* ATCC 367 was extracted using an IBI genomic DNA mini kit (MidSci, St. Louis, MO, USA) according to the manufacturer's instructions. The *xylAB* operon from *L. brevis* ATCC 367's genome was amplified by PCR using *xylAB*-F and *xylAB*-R primers, which were designed based on the sequence of the *xylAB* operon derived from *L. brevis* ATCC 367's genome (GenBank accession number NC_008497.1). The amplified 2.98 kb DNA fragment was then digested with *Xho*I and *Pvu*II and ligated into *Xho*I and *EcoR*V digested pLEM415 vector. The promoter *clpC* fragment from the pCU-P*xylAB* vector was amplified by PCR using *clpC*-F and *clpC*-R primers, the amplified fragment was then ligated into the pLEM415 vector harboring the *xylAB* operon using recombinant sites *Kpn*I and *Xho*I. The resulting plasmid designed to express xylose isomerase and xylulokinase under control of the *clpC* promoter was designated pLEM415-*xylAB* (Fig.6.1) and was sent to Molecular Cloning Laboratories (South San Francisco, CA, USA) for sequencing. The sequence thus obtained was verified for in-frame cloning using MEGA 6 (Tamura et al. 2013).

pLEM415-*xylAB* was then transformed into *L. plantarum* NCIMB 8826 Δ*ldhL1* using the method modified from Narita et al. (2006). *L. plantarum* NCIMB 8826 Δ*ldhL1* was cultivated overnight in a test tube containing 5 mL of MRS broth, and the overnight culture was then diluted 100 times with fresh MRS broth and cultivated at 37 ℃ until the OD_{600} value reached 0.5 to 0.8. Cells were washed five times with wash buffer (272 mM sucrose, 7 mM HEPES, 1mM $MgCl_2$, pH 7.4), and suspended in 1 mL electroporation buffer (wash buffer with 20 % (w/v) PEG6000). Fifty microliters of competent cells were mixed with 0.1~0.3 μg of plasmid DNA and incubated on ice for 30 min. Before electroporation, 1 μL of TypeOne Restriction Inhibitor (Epicentre Technologies Corp. Madison, WI, USA) was added. Samples were then subjected to a

2.5 kV, 25 µFD and 200 Ohm electric pulse in a 0.2 cm cuvette by using a Gene pulser Xcell electroporator (Bio-Rad, Hercules, CA, USA). Fresh MRS broth (500 µL) was immediately added, and cells were incubated for 2 h at 37 ℃ before plating on MRS agar supplemented with 25 µg mL^{-1} erythromycin. Plates were incubated at 37 ℃ for 2 to 3 days. The resulting transformant was designated *Lactobacillus plantarum* NCIMB 8826 Δ*ldhL1*-pLEM415-*xylAB* and used in the fermentation experiments.

D-lactic acid production from pure sugars

A fed-batch xylose fermentation experiment was conducted in a 7 L fermenter with 5 L working volume (Bioflo 110, New Brunswick Scientific Inc., Enfield, CT, USA). *L. plantarum* NCIMB 8826 Δ*ldhL1*-pLEM415-*xylAB* was grown in MRS broth with 25 µg mL^{-1} erythromycin until OD value reached about 5, and was used to inoculate at 5 % (v/v) to the fermenter containing 5 L modified MRS medium with 40 g L^{-1} of xylose supplemented with 10 g L^{-1} of peptone, 5 g L^{-1} of YE, 2 g L^{-1} of ammonium citrate, 2 g L^{-1} of K_2HPO_4, 0.1 g L^{-1} of $MgSO_4.7H_2O$, and 0.05 g L^{-1} of $MnSO_4.4H_2O$. Temperature was controlled at 37 ºC with agitation of 150 rpm. The pH was maintained at 6.5 by adding 10 N sodium hydroxide.

A mixed sugars fermentation experiment was conducted in a 2-L fermenter with 1.5 L working volume (Biostat. B, Satorius AG, Goettingen, Germany). Fermentation medium contained 37.5 g L^{-1} glucose, and 19.7 g L^{-1} xylose and was supplemented with 10 g L^{-1} of peptone, 5 g L^{-1} of YE, 2 g L^{-1} of ammonium citrate, 2 g L^{-1} of K_2HPO_4, 0.1 g L^{-1} of $MgSO_4.7H_2O$, and 0.05 g L^{-1} of $MnSO_4.4H_2O$. Fermentation conditions were identical to the xylose fermentation experiment.

D-lactic acid production from corn stover

Sequential saccharification and fermentation (SHF), and simultaneous saccharification and fermentation (SSF) experiments with corn stover were carried out in 150-mL conical flasks. In SHF experiments, 2 g of dried alkali-treated corn stover was hydrolyzed by Cellic CTec2 obtained from Novozyme. Inc. (Franklinton, NC, USA). The dosage of CTec2 was added at 8 FPU per gram of corn stover. Saccharification was carried out at 50 ℃ for 48 h, and centrifuged at 10.000 × g for 10 min (Sorvall RC 5C Plus, GMI Inc., MN, USA). Supernatant was collected and pH adjusted to 6.5 by sodium hydroxide. Corn stover hydrolysate was supplemented with all the components (except sugars) of the modified MRS medium to make final volume of 50 mL,

and 3 % (w/v) of $CaCO_3$ was added to buffer the pH. Fermentation was performed at 37 ℃ with 150 rpm agitation.

In SSF experiments, 2 g of alkali-treated corn stover was supplemented with all the components (except sugars) of the modified MRS medium and volume was adjusted to 50 mL by 50 mM sodium citrate buffer (pH 5). Cellic CTec2 was added at 8 FPU per gram of corn stover and *L. plantarum* NCIMB 8826 Δ*ldhL1*- pLEM415-*xylAB* inoculum was added and 5 % (v/v). SBME was evaluated to substitute YE for D-lactic acid production from corn stover via SSF process. SBME was added at 10 % (v/v) with 5 g L^{-1} peptone, 2 g L^{-1} of ammonium citrate, 2 g L^{-1} of K_2HPO_4, 0.1 g L^{-1} of $MgSO_4.7H_2O$, and 0.05 g L^{-1} of $MnSO_4.4H_2O$. Fermentation conditions were the same as describe in SHF experiments.

The fed batch SSF experiment was carried out in 500-mL conical flasks with working volumes of 100 mL. Dried alkali-treated corn stover (4 g), Cellic CTec2 (5.6 FPU g^{-1} of corn stover), SBME (15 % v/v), peptone (3 g L^{-1}), salts (2 g L^{-1} of ammonium citrate, 2 g L^{-1} of K_2HPO_4, 0.1 g L^{-1} of $MgSO_4.7H_2O$, and 0.05 g L^{-1} of $MnSO_4.4H_2O$), and inoculum (5 % v/v) were added at the beginning of fermentation. $CaCO_3$ (3 g) was also added in the beginning to maintain pH in the flasks. Feed was applied every 36 h, which contained 2 g of corn stover, 1.5 g of $CaCO_3$, and 15 mL of SBME along with Cellic CTec2 at a dosage of 5.6 FPU g^{-1} of corn stover.

Statistical Experimental Design

Response surface methodology was used to optimize key factors affecting lactic acid production, which were enzyme loading and, SBME and peptone concentrations in a batch shake-flask. Design Expert V. 8.0.7.1 (Stat-Ease Inc., Minneapolis, MN, USA) was used to generate experimental design, assess the response of dependent variables and also generate response surface plots.

Three independent factors (enzyme loading, SBME, and peptone concentration) and their respective levels are given in Table 6.2. Box-Behnken design (Box and Behnken 1960) was adopted to optimize the levels of these three factors. A total of 17 runs comprising 5 replicates in the central point were carried out in random order. Lactic acid concentration was the response. A second-order quadratic model was fitted for the experimental results. Validation of optimized conditions was carried out with four replications.

Analytical procedures

Cell growth was measured by a spectrophotometer at a wavelength of 600 nm (UV-1650PC, Shimadzu, Torrance, CA, USA). Concentrations of lactic acid, acetic acid, glucose, and xylose were measured using a high-performance liquid chromatography (HPLC) system equipped with a refractive index detector (RID-10A) and a Rezex ROA organic acid column (300×7.8 mm, Phenomenex Inc., Torrance, CA, USA). Samples were centrifuged at a speed of 15,000 ×g for 10 min (Eppendorf, Hauppauge, NY, USA), and the supernatant was acidified with 1 N H_2SO_4 and centrifuged at 15,000 ×g for 15 min to remove $CaSO_4$ precipitant. Supernatant was diluted 10 times with deionized water before analysis. 0.005 N H_2SO_4 was used as mobile phase at an elution speed of 1 mL min^{-1}, column temperature was maintained at 80 ℃, and RID detector temperature was maintained at 40 ℃. The optical purity of lactic acid was measured by the method described by Zhang and Vadlani (2013).

Results

Lactic acid fermentation from pure sugars using L. plantarum NCIMB 8826 ΔldhL1-pLEM415-xylAB

Xylose-assimilating genes (*xylAB*) transformed strains were selected on an MRS plate containing 25 μg mL^{-1} of erythromycin. Cultivation with xylose as the sole carbon source was carried out using *L. plantarum* NCIMB 8826 Δ*ldhL1* as a control to confirm the transformation strains were able to use xylose. After 48 h of cultivation, the OD_{600} of *L. plantarum* NCIMB 8826 Δ*ldhL1* was 0.7, but *L. plantarum* NCIMB 8826 Δ*ldhL1*-pLEM415-*xylAB* showed remarkably increased growth (OD_{600} was 3.6); these results indicated that introduction of *xylAB* genes into *L. plantarum* NCIMB 8826 Δ*ldhL1* resulted in successful assimilation of xylose by *L. plantarum* NCIMB 8826 Δ*ldhL1*-pLEM415-*xylAB*.

Fermentation with 40 g L^{-1} of xylose was performed to evaluate the lactic acid fermentation ability of *L. plantarum* NCIMB 8826 Δ*ldhL1*-pLEM415-*xylAB* from pure xylose. As shown in Fig. 6.2A, 19.7 g L^{-1} of D-lactic acid was produced along with 12.8 g L^{-1} of acetic acid at the end of the first stage of fermentation. The yield of D-lactic acid from xylose in the first stage was 0.53 g g^{-1}, which was comparable to that of *L. brevis* (0.50 g g^{-1}). After 56 h of fermentation, 700 mL of fermentation broth was pumped out and 700 mL of fresh medium

containing 200 g xylose was added and fermentation continued for next 112 h. In the end of fermentation 30.1 g L^{-1} of D-lactic acid was produced with 20.5 g L^{-1} of acetic acid.

A mixed sugars experiment using glucose and xylose at a 2:1 ratio was conducted to mimic sugar composition in enzymatic hydrolysate of alkali-treated corn stover. As shown in Fig. 6.2B, almost all glucose was consumed at 36 h, whereas xylose was consumed more slowly than glucose and almost all xylose was consumed at 48 h. At the end of fermentation, 47.2 g L^{-1} of D-lactic acid and 8.9 g L^{-1} of acetic acid were obtained. Yield of lactic acid from both glucose and xylose was 0.84 g g^{-1} and productivity was 0.98 g L^{-1} h^{-1}. Simultaneous utilization of glucose and xylose by *L. plantarum* NCIMB 8826 Δ*ldhL1*-pLEM415-*xylAB* in this study greatly increased lactic acid productivity compared with a co-culture system in which *L. brevis* was sequentially cultivated after glucose was almost consumed by *L. plantarum* and resulted in lactic acid yield of 0.85 g g^{-1} and productivity of 0.51 g L^{-1} h^{-1} (Zhang and Vadlani 2015).

D-Lactic acid production from corn stover

Fig. 6.3A shows the fermentation profile of using corn stover hydrolysate. Glucose (16.3 g L^{-1}) was consumed within 12 h, and xylose (8.6 g L^{-1}) was consumed within 24 h. 19.4 g L^{-1} of D-lactic acid along with 4.6 g L^{-1} of acetic acid was produced. The yield of D-lactic acid from total sugar was 0.78 g g^{-1}, and productivity was 0.27 g L^{-1} h^{-1}. The SSF process was applied to convert corn stover to D-lactic acid to improve lactic acid productivity (Fig. 6.3B). Glucose and xylose concentration in the medium were maintained less than 1 g L^{-1} during the entire process. As shown in Table 6.3, D-lactic acid concentration reached up to 26.8 g L^{-1} with overall yield of 0.67 g g^{-1} and productivity of 0.74 g L^{-1} h^{-1}. D-lactic acid yield and productivity from corn stover by this recombinant strain were greatly improved compared with D-lactic acid yield (0.50 g g^{-1}) and productivity (0.32 g L^{-1} h^{-1}) from corn stover by homofermentative strain *L. delbrueckii* in our previous study (Zhang and Vadlani 2013).

Optimization of parameters affecting D-lactic acid production using RSM

A Box-Behnken design with 17 runs was conducted to evaluate the effect of enzyme loading, and peptone and SBME concentrations on D-lactic acid production via the SSF process. Maiti et al. (2011) also applied a Box-Behnken design to optimize process parameters for ethanol production, because it required fewer experimental runs than central composite design. Fig. 6.3C shows the fermentation profile of the SSF experiment with Cellic CTec2 at 8 FPU g^{-1} of corn

stover, 5 g L^{-1} peptone, and 10 % (v/v) SBME; these data were used as the basis (0 level) of the statistical experimental design. Utilization of glucose and xylose in SBME as a substitute for YE in the experiment were slower compared with using YE and a high concentration of peptone (Fig. 6.3B). Glucose accumulated to maximum level of 4.6 g L^{-1} at 6 h, and maintained lower than 1 g L^{-1} after 12 h. Xylose consumption was slower compared with that of glucose. Xylose accumulated to a maximum concentration of 3.9 g L^{-1} at 12 h, and slowly reduced to 1.3 g L^{-1} at 36 h. At the end of fermentation, 29.4 g L^{-1} lactic acid along with 5.1 g L^{-1} of acetic acid were obtained (Table 6.3).

Table 6.4 shows the ANOVA of the second order response surface model for lactic acid production. The model F-value of 9.04 indicated the model was significant, the chance (p-value) that a "Model F-value" this large would occur as a result of noise was only 0.42 %. The coefficient estimates and their corresponding p-values were all less than 0.05, suggesting that all the variables were significant. The equation used to demonstrate lactic acid production in coded terms was:

$$D\text{-}lactic\ acid(g/L) = 29.36 + 2.56 \times A + 3.04 \times B + 2.13 \times C - 0.68 \times A \times B - 0.05 \times A \times C - 0.65 \times B \times C - 0.27 \times A^2 - 2.37 \times B^2 + 0.46 \times C^2$$

. The R^2 value was used to judge if the model is a good fit or not (Liu and Wang 2007), R^2 for the above equation was 0.92, which indicates that 92 % of the variation in lactic acid production can be explained by this model. All three variables positively influenced lactic acid production, which means higher level of all three variable would result higher lactic acid production.

Numerical optimization in which the optimal conditions were generated by setting goals for each response; was chosen to maximize lactic acid production and minimize enzyme loading and peptone concentration. D-lactic acid concentration was set at a range of 27 to 32 g L^{-1} in order to achieve D-lactic acid production equivalent to that using YE, which was 26.8 g L^{-1}. The highest desirability was 0.446, at which the optimal conditions were enzyme loading (5.6 FPU g^{-1}), peptone (3 g L^{-1}), and SBME (15 % v/v). Validation experiments based on four replicates gave lactic acid production of 29.9 g L^{-1}, which was within the 95 % confidence level of the predicted value (25.5 to 32.8 g L^{-1}). After optimization, the enzyme loading decreased 30 %, and peptone concentration decreased 70 %, and YE was completely replaced by SBME.

Fed-batch experiment using alkali treated corn stover via SSF

In accordance with the statistical study and taking production costs into consideration, the optimal conditions (enzyme loading 5.6 FPU per g of corn stover, 3 g L^{-1} of peptone and 15 % (v/v) SBME) were evaluated in fed-batch experiment. Four batches of alkali treated corn stover were fed at 36, 72, 108 and 144 h giving a total amount of 12 g corn stover. Fig. 6.4 shows the concentration of lactic acid and acetic acid in the fed-batch experiment. The abrupt drops at feeding point (36, 72, 108 and 144 h) were due to the volume change caused by taking samples and adding fresh corn stover and SBME. D-Lactic acid concentration increased rapidly within the first 72 h, then leveled off after 192 h and slightly decreased to 59.5 g L^{-1}, but acetic acid concentration gradually increased to 13.2 g L^{-1}. Because D-lactic acid is the product of interest in this study, the data point at 192 h was used to calculate yield and productivity. The overall yield of lactic acid was 0.77 g g^{-1} and productivity was 0.32 g L^{-1} h^{-1} (Table 6.3).

Discussion

In this study, we constructed a recombinant plasmid used for xylose assimilation under the control of a constitutive *clpC* promoter and transformed *L. plantarum* NCIMB 8826 Δ*ldhL1* with this plasmid. D-lactic acid production from corn stover and SBME as a substitute for YE by the resulting recombinant strain was also demonstrated. At first, two constitutive promoters, one from lactate dehydrogenase genes (*ldh*) of *L. casei* (GenBank accession number M76708) and the other one from S-layer protein gene (*slpA*) of *L. brevis* (GenBank accession number Z14250), were ligated upstream of the *xylAB* operon in the pLEM415 plasmid. Disruption of plasmids was observed during cloning in *E.coli*; such plasmid instability is possibly owing to promoters from LAB, because the pLEM415 plasmid carrying *xylAB* without a promoter sequence was stable in *E.coli*. The *clpC* promoter from a polypeptide similar to previously characterized ClpC ATPase chaperones of *Lactobacillus fermentum* BR11 (McCracken et al. 2000; Lazazzera and Grossman 1997) was used to construct an expression vector pCU by Okano et al. (2009a). Interestingly, plasmid instability was not observed during cloning of the *clpC* promoter in *E.coli* in our study. The possible explanation could be that high-level expression of the *xylAB* gene is detrimental to *E.coli* (Posno et al. 1991b). The *clpC* promoter is reported to be negatively regulated by CtsR, a product of *orf1* gene (Derré et al. 1999). A homologous protein in *E.coli* may act as a repressor for *clpC* promoter results in lower-level expression of *xylAB* genes compared with *slpA* and *ldh*

promoters in *E.coli* cells. The recombinant pLEM415-*xylAB* plasmid under control of the *clpC* promoter was used to transform *Lactobacillus plantarum* Δ*ldhL1*, and a homofermentative strain *Lactobacillus delbrueckii* ATCC 9649, which was used in our previous study (Zhang and Vadlani 2013). Unfortunately, electroporation attained a lack of *L. delbrueckii* tranformants, which may be attributed to various restriction modification systems encoded by the host (Rixon and Warner 2003), or the TypeOne restriction inhibitor failing to improve the transformation efficiency of *L. delbrueckii* in our study.

L. plantarum NCIMB 8826 Δ*ldhL1*-pLEM415-*xylAB* produced highly optically pure D-lactic (~99.7 %) from either refined sugars or lignocellulosic biomass in this study. The high optical purity is preferred for production of poly lactic acid, which usually requires 99 % or greater purity. D-lactic acid yield from xylose by *L. plantarum* NCIMB 8826 Δ*ldhL1*-pLEM415-*xylAB* at the first stage was 0.53 g g^{-1}, which is close to the yield (0.57 g g^{-1}) obtained by using *L. plantarum* NCIMB 8826 Δ*ldhL1* harboring the pCU-P*xylAB* plasmid (Okano et al. 2009a). At the second stage of xylose fermentation, xylose was consumed very slowly, and 12.9 g L^{-1} was left at the end of fermentation. The slow consumption of xylose probably occurred because of exhaustion of other nutrients such as metal ions (Mn^{2+}, Mg^{2+}) and vitamins, which serve as activators in enzymatic reactions (Yadav et al. 2011). Similar to *L. plantarum* NCIMB 8826 Δ*ldhL1*-pCU-P*xylAB*, *L. plantarum* NCIMB 8826 Δ*ldhL1*-pLEM415-*xylAB* did not show a hierarchical sugar utilization pattern in the mixed sugar fermentation. D-lactic acid yield of 0.84 g g^{-1} was obtained, which was slightly higher than the yield (0.76 g g^{-1}) obtained by using *L. plantarum* NCIMB 8826 Δ*ldhL1*-pCU-P*xylAB* in mixed sugar fermentation (Yoshida et al. 2011).

Production of lactic acid from corn stover via the SSF process has been described using various wild-type LAB (Zhang and Vadlani 2013; 2015; Zhu et al. 2007). The SSF process is widely used in the bio-ethanol industry and producing chemicals from lignocellulosic biomass by increasing the productivity and reducing feedback inhibition that usually occurs in the hydrolysis step is a much more effective method. The low concentration of glucose and xylose during the SSF process (Fig. 6.3) indicated the consumption of sugars by bacteria was much more rapid than release of sugars from the hydrolysis step, which also was observed in our previous study (Zhang and Vadlani 2013; 2015). D-lactic acid yield increased to 16 % compared to the yield obtained by using homo-fermentative strain *L. delbrueckii* (Zhang and Vadlani 2013), and 72 %,

compared to the yield obtained by using the xylose assimilation genes provider strain *L. brevis* (Zhang and Vadlani 2015).

Lactic acid bacteria are nutritionally fastidious, and using expensive nitrogen sources such as YE and peptone has been common to spur fast growth of lactic acid bacteria and lactic acid production. SBME is a promising low-cost nitrogen source alternative that completely replaced YE for D-lactic acid production in our study. Replacing YE with 10 % (v/v) SBME did not significantly change lactic acid production; in fact, it increased slightly (Table 5.3), probably because of the presence of certain strong growth stimulators for LAB in SBME, such as glutamic acid and glutamine (Batal et al. 2000; Maxwell et al. 1942). Fed-batch fermentation, an industrially preferred fermentation method was applied to improve lactic acid concentration in this study. Lactic acid concentration stopped increasing after 192 h; the cessation is probably owing to the lack of an available carbon source, as evidence by the lower concentration of sugars. The other reason may be the inhibition of high concentration of lactic acid. Bustos et al. (2005) reported that *Lactobacillus pentosus* ATCC 8041 was strongly inhibited when lactic acid concentration reached 46 g L^{-1}. The decrease in lactic acid after 192 h was probably due to further convertion of lactic acid to acetic acid and CO_2 by lactate oxidase when oxygen is present (Kandler 1983). Zhu et al. (2007) reported production of lactic acid from corn stover and substitute YE with corn steep liquor using *Lactobacillus pentosus* ATCC 8041 via fed-batch SSF, and lactic acid yield reached 65 % of the maximum theoretical yield (assuming assimilation of glucose follow EMP, and assimilation of xylose and arabinose following PK pathway). The glucan and xylan contents of corn stover in this study were estimated to be 53.9 % (w/w) and 29.7 % (w/w), respectively (Guragain et al. 2013); thus, 12 g alkali-treated corn stover containing 7.2 g of glucose and 4.1 g of xylose which were calculated using the following equations: $glucose\ (g) = glucan\ (g)/0.9$, and $xylose\ (g) = xylan\ (g)/0.88$. The yield in our study was calculated to be 95 % of the maximum theoretical yield. The productivity of lactic acid was 0.05 g L^{-1} h^{-1} in the previous study, whereas productivity in our study was 0.32 g L^{-1} h^{-1} because of simultaneous use of glucose and xylose by *L. plantarum* NCIMB 8826 Δ*ldhL1*-pLEM415-*xylAB*, whereas the xylose consumption occurred only after depletion of glucose in Zhu et al.'s study (2007).

Conclusions

In conclusion, this study demonstrated D-lactic acid production from corn stover and SBME using *L. plantarum* NCIMB 8826 Δ*ldhL1*-pLEM415-*xylAB*. Our findings show that *L. plantarum* NCIMB 8826 Δ*ldhL1*-pLEM415-*xylAB* was able to simultaneously use all the major sugars derived from corn stover to produce D-lactic acid with high optical purity (>99 %). This fermentation process is a value added approach to producing D-lactic acid with high optical purity from lignocellulosic biomass and a low-cost nitrogen source, which could reduce the manufacturing cost of lactic acid production.

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Table 6.1 Bacterial strains and plasmids

Strains and plasmids	Relevant characteristics	Antibiotic resistance	Reference or source
Strains			
Escherichia coli DH5α	lacZ ΔM15, recA1, endA1 mutations		Invitrogen
L. plantarum NCIMB 8826 Δ*ldhL1*	*L.plantarum* NCIMB 8826 L-lactate dehydrogenase gene1 was disrupted		Okano et al. 2009c
L.brevis	Source of *xylAB* gene		ATCC
Δ*ldhL1*-pLEM415-*xylAB*	*L. plantarum* NCIMB 8826 Δ*ldhL1* strain carrying pLEM415-*xylAB* plasmid		This study
Plasmids			
pLEM415	*Escherichia coli- Lactobacillus* shuttle vector	Erythromycin Ampicillin	Serror et al. 2002
pCU-P*xylAB*	Expression vector containing the *xylAB* operon under the control of *clpC* UTLS promoter	Erythromycin	Okano et al. 2009b
Primers			
xylAB-F	TAACTCGAGGGAGGGCTTTTATAATTATGAC		
xylAB-R	TAACAGCTGCTAAAGCTCCGCTCGCCGATAGTCTAA		
clpC-F	GCGGTACCCTTAAAATATAGTCATAGAATTAGGGCG		
clpC-R	GCCTCGAGTAATCTTGACCATTATTTTACCACACTT		

* restriction enzyme cleavage sites are underlined

Table 6.2 Factors and levels of variables in the Box-Behnken design

			Coded levels	
Factor		-1	0	1
A	Enzyme loading (FPU g^{-1} corn stover)	4	8	12
B	Peptone (g L^{-1})	0	5	10
C	SBME (% v/v)	5	10	15

Table 6.3 D-lactic acid production from corn stover

	D-lactic acid ($g\ L^{-1}$)	Acetic acid ($g\ L^{-1}$)	Overall yield[1] ($g\ g^{-1}$)	Productivity[2] ($g\ L^{-1}\ h^{-1}$)
SHF	19.4 ± 0.3^{C}	4.6 ± 0.0^{C}	0.49 ± 0.01^{C}	0.40 ± 0.01^{B}
SSF+YE	26.8 ± 1.0^{B}	6.2 ± 0.8^{B}	0.67 ± 0.02^{B}	0.74 ± 0.03^{A}
SSF+SBME	29.4 ± 0.8^{B}	5.1 ± 0.3^{BC}	0.73 ± 0.02^{AB}	0.82 ± 0.02^{A}
Fed batch	61.4 ± 1.1^{A}	12.5 ± 0.2^{A}	0.77 ± 0.02^{A}	0.32 ± 0.01^{C}

Each mean is based on three replications ($p < 0.05$). Values with same letters in the same column are not significantly different.

[1]Overall yield was calculated by dividing the amount of D-lactic acid by the amount of biomass used.

[2]Productivity was defined as the amount of D-lactic acid produced per liter per hour. In SHF, the 48 h hydrolysis time was included in productivity calculation.

Table 6.4 ANOVA for response surface quadratic model for lactic acid production

Source	Sum of Squares	df	Mean Square	F value	p-value Prob > F
Model	190.65	9	21.18	9.04	0.0042
A-enzyme loading	52.53	1	52.53	22.41	0.0021
B- peptone	73.81	1	73.81	31.49	0.0008
C-SBME	36.13	1	36.13	15.41	0.0057
AB	1.82	1	1.82	0.78	0.4072
AC	0.010	1	0.010	0.004266	0.9498
BC	1.69	1	1.69	0.72	0.4239
A^2	0.30	1	0.30	0.13	0.7305
B^2	23.6	1	23.60	10.07	0.0156
C^2	0.88	1	0.88	0.38	0.5592

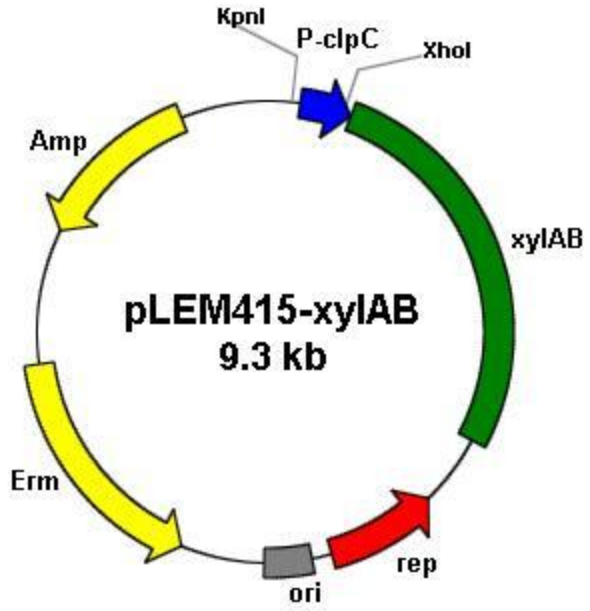


Figure 6.1 Recombinant pLEM415-*xylAB* plasmid

xylAB: xylose assimilation operon from *L. brevis* ATCC 367; ori: origin of replication; rep: replication gene from pLEM3 (Fons et al. 1997); Erm: erythromycin resistance gene from pLEM3; Amp: ampicillin resistance gene from pBII(SK^+); P-clpC: *clpC* promoter from pCU-P*xylAB*

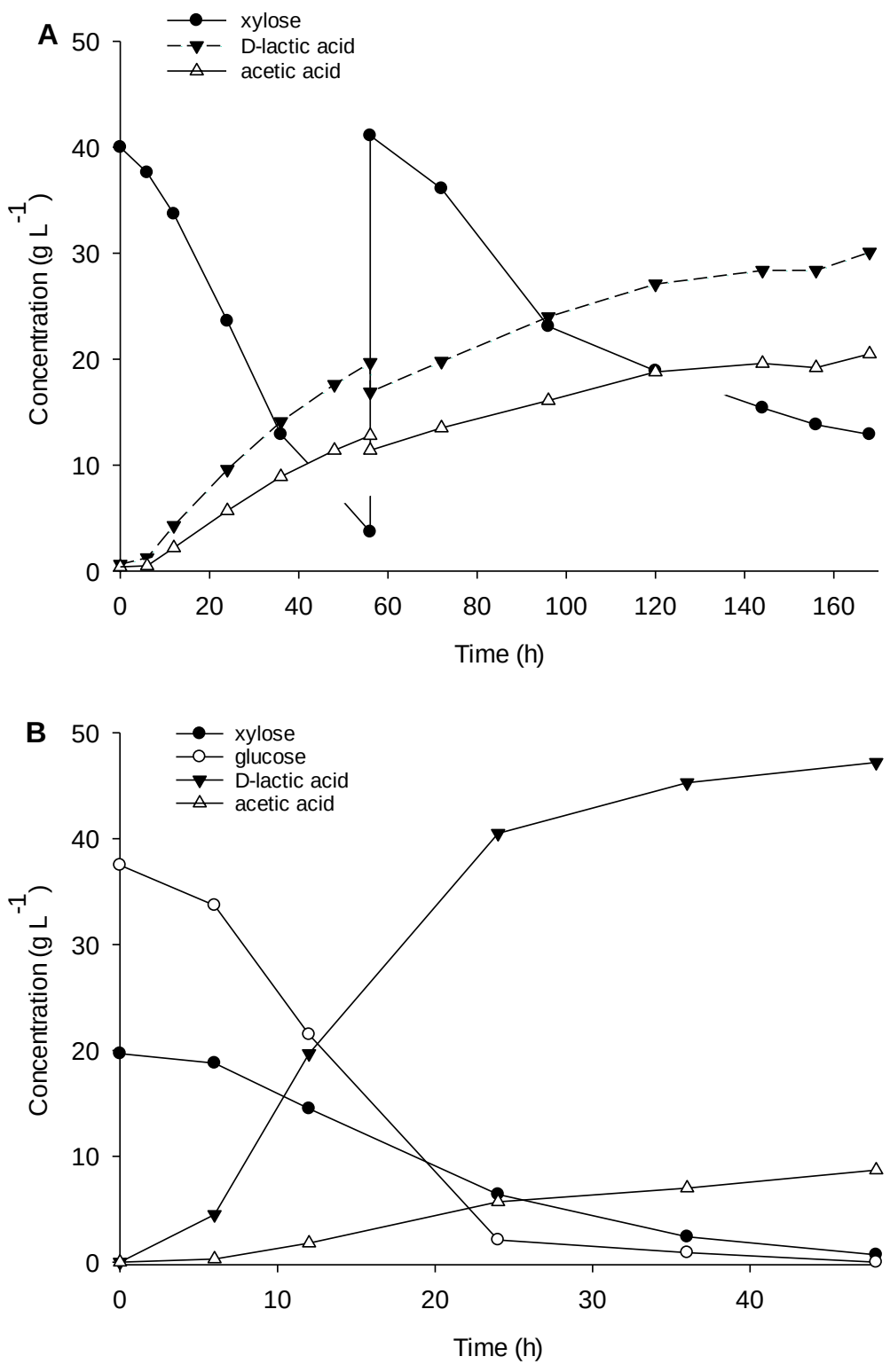
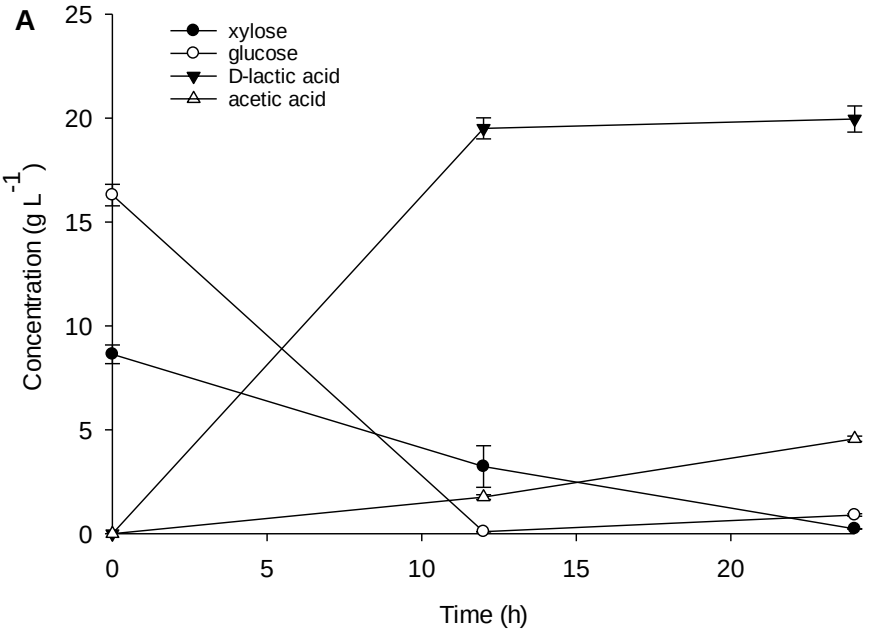


Figure 6.2 D-lactic acid production from pure sugars: (A) Xylose; (B) mixture of xylose and glucose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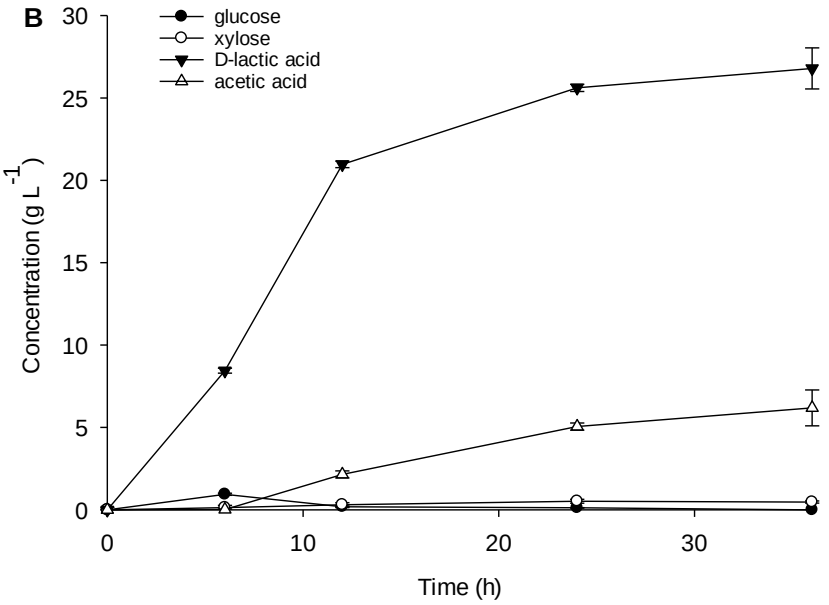
xylose
glucose
D-lactic acid
acetic acid
Concentration (g L-1)
Time (h)
0
5
10
15
20
25


B
glucose
xylose
D-lactic acid
acetic acid
Concentration (g L-1)
Time (h)
0
5
10
15
20
25
30

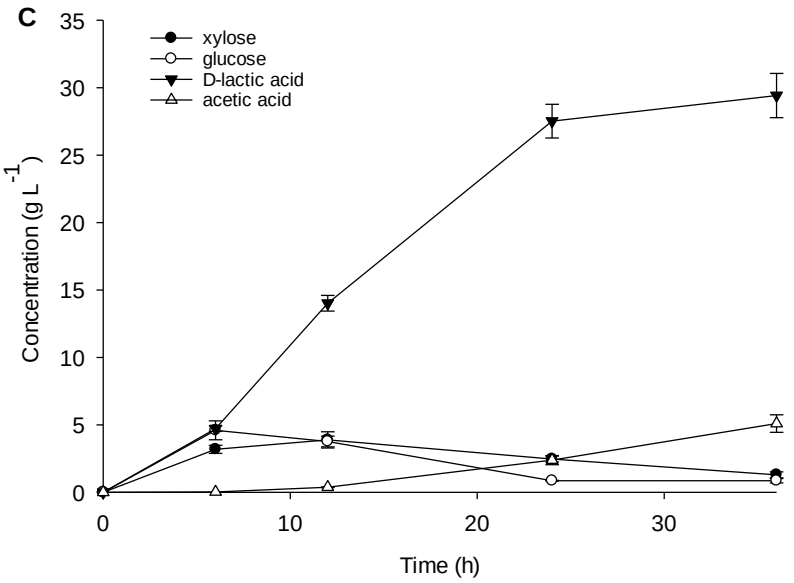


Figure 6.3 D-lactic acid production from corn stover via (A) SHF; (B) SSF with YE supplement; (C) SSF with SBME supplement

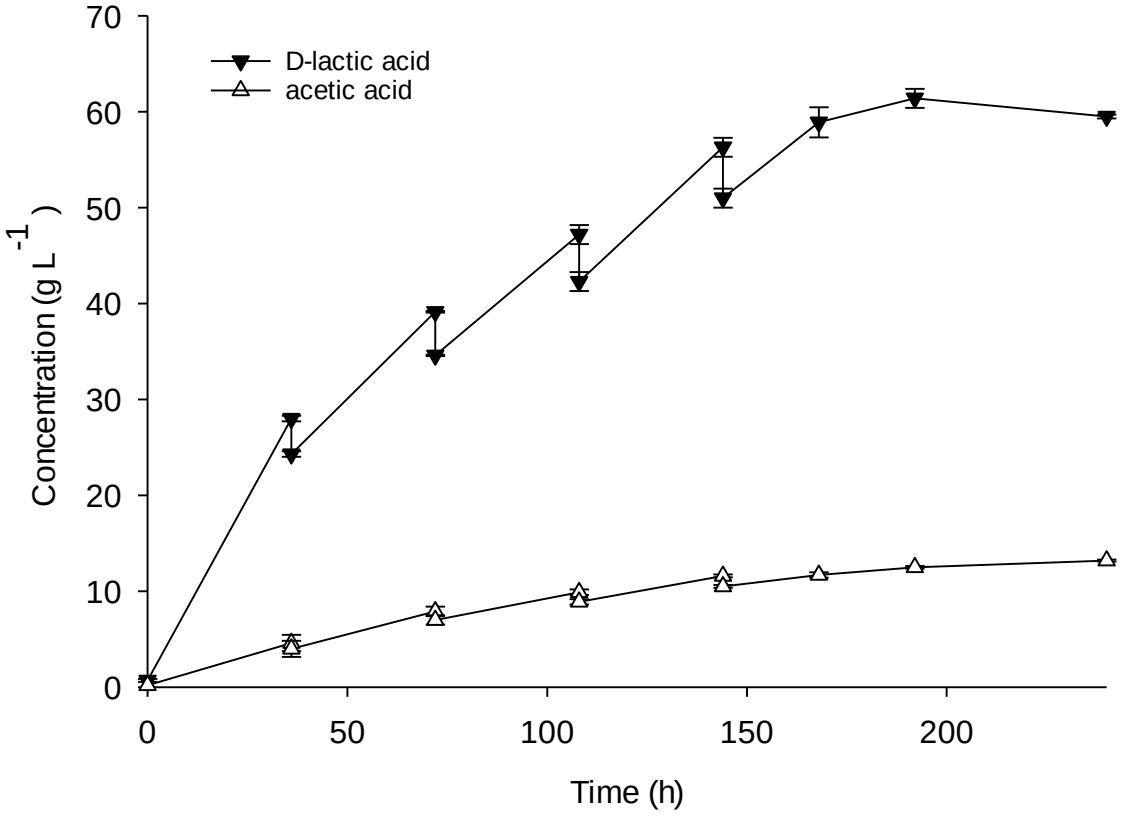


Figure 6.4 Lactic acid and acetic acid concentration in fed-batch SSF experiments

Chapter 7 - Metabolic flux analysis of carbon balance in *Lactobacilli* strains

Abstract

Metabolic flux analyses were calculated based on the carbon balance of *Lactobacillus* strains used in this study. Results confirmed that *L brevis* metabolized both glucose and xylose through PK pathway, but the end-products ratio varied when different carbon source were used. *L. delbrueckii*, *L. plantarum* ATCC 21028, *L. plantarum* NCIMB 8826 Δ*ldhL1, L. plantarum* NCIMB 8826 Δ*ldhL1*-pCU-P*xylAB* and *L. plantarum* NCIMB 8826 Δ*ldhL1*-pLEM415-*xylAB* metabolized glucose via EMP. Xylose was metabolized through PK pathway in *L. brevis*, *L. plantarum* NCIMB 8826 Δ*ldhL1*-pCU-P*xylAB* and *L. plantarum* NCIMB 8826 Δ*ldhL1*-pLEM415-*xylAB*. Our analysis showed that in the presence of xylose, a small amount of glucose was channeled into PK pathway in *L. plantarum* NCIMB 8826 Δ*ldhL*1-pCU-P*xylAB* and *L. plantarum* NCIMB 8826 Δ*ldhL1*-pLEM415-*xylAB*.

Introduction

Raw material is a key factor that decides the production cost of lactic acid. It's estimated to be 30 to 40 % of the total cost (Oh et al. 2005). Lactic acid can be produced from different raw materials including starchy and sugar based waste from food industry, municipal solid waste such as waste paper products, and agricultural residues. Lactic acid bacteria can convert carbohydrate in the above mentioned raw materials into lactic acid and/or other products depending on their metabolic pathway. Generally, homofermentative lactic acid bacteria convert carbohydrate into lactic acid using the Embden-Meyerhof pathway (EMP), whereas heterofermentative lactic acid bacteria metabolize carbohydrate through the phosphoketolase (PK) pathway to produce lactic acid, acetic acid, ethanol, formic acid and CO_2. Pentose sugars are usually metabolized through the PK pathway. By understanding the distribution of carbon flux in lactic acid bacteria, useful information can be gathered to enable proper selection of strains for desired level of lactic acid production with different raw materials.

In this doctoral research, six *Lactobacillus* strains were studied to produce lactic acid from different lignocellulosic biomass, viz. *Lactobacillus delbrueckii* ATCC 9649, *Lactobacillus brevis* ATCC 367, *Lactobacillus plantarum* ATCC 21028, *Lactobacillus plantarum* NCIMB

8826 Δ*ldhL1* and its two derivatives: *Lactobacillus plantarum* NCIMB 8826 Δ*ldhL1*-pCU-P*xylAB* and *Lactobacillus plantarum* NCIMB 8826 Δ*ldhL1*-pLEM415-*xylAB*. *L. delbrueckii* is a homofermentative strain which can metabolize most hexose sugars including glucose and fructose through the EMP pathway to produce D-lactic acid, but xylose cannot be used by this strain (Demirci et al. 1992; Zhang and Vadlani 2013; Calabia and Tokiwa 2007). *L. brevis* can use both glucose and xylose simultaneously to produce a racemic mixture of D/L lactic acid (Kim et al. 2009; Zhang and Vadlani 2015). *L. plantarum* processes glucose through the EMP pathway to produce a racemic mixture of D/L lactic acid, but it also possesses an inducible PK pathway when pentose sugars such as arabinose are present. However, this strain cannot use xylose (Vadlani et al. 2008a, 2008b; Zhang and Vadlani 2015). *L. plantarum* NCIMB 8826 was genetically modified to produce only D-lactic acid by deletion of the L-lactate dehydrogenase gene, and the resulting strain is designated as *Lactobacillus plantarum* NCIMB 8826 Δ*ldhL1* (Okano et al. 2009c). Δ*ldhL1* is able to use arabinose (Okano et al. 2009a), but it cannot use xylose. By introduction of xylose assimilation plasmids into this strain, the resulting Δ*ldhL1*-pCU-P*xylAB* and Δ*ldhL1*-pLEM415-*xylAB* strains produced D-lactic acid from both glucose and xylose. The purpose of this study was to investigate detailed carbon metabolic flux in these six *Lactobacillus* strains using different carbon source. Metabolic flux analysis results could provide useful information about strain selection, distribution of carbon fluxes, and thereby means to minimize byproduct formation, when different raw materials are used for lactic acid production.

Materials and methods

Fermentation conditions

Glucose (30 g L^{-1}), xylose (30 g L^{-1}) and mixed sugar fermentation (30 g L^{-1} glucose and 15 g L^{-1} xylose) experiments were conducted in 100 mL sealed serum bottles with 50 mL working volume for *L. delbrueckii, L. brevis, L. plantarum*, Δ*ldhL1,* Δ*ldhL1*-pCU-P*xylAB* and Δ*ldhL1*-pLEM-P*xylAB*. Calcium carbonate was added at 3 % (w/v) to buffer the pH. Temperature was kept at 37℃ and agitation rate was kept at 150 rpm.

Sample analysis

Glucose, xylose, lactic acid, acetic acid and ethanol were measured via HPLC system using a refractive index detector (RID) and a Rezex ROA organic acid column (300×7.8 mm,

Phenomenex Inc., Torrance, CA). Samples were eluted using 0.005 N sulfuric acid at a flow rate of 1 mL min^{-1}.

Flux analysis equation development

The method developed by Ohara et al. (2007) was used for calculating the carbon metabolic flow. Fig. 7.1 shows the proposed EMP and PK pathway of *Lactobacillus* strains. Based on Fig. 7.1 The junctions A to I were set; the same letters were organized in a circuit diagram and each pathway was named as f_1 to f_{17} (Fig. 7.2). The f_n (the amount of carbons in each pathway) was equal to the amount of molecules in the flow multiplied by the number of carbons in the molecules. For example the equation applied to junction B in Fig. 7.2 was: $f_2 - f_3 - f_4 = 0$; similar equations for other junctions were formed in the same way. The molecular rate of inflow and outflow was assumed to be constant in junction B, F and I, thus the amount of molecules were calculated by dividing f_n by the number of carbons in the molecules. Concerning the outflow of molecules from junction B, equation can be formed as $f_3/3 = f_4/2$. Similar equations can be formed for outflow of molecules from junction F and I. All of the equations were expressed in an augmented matrix and f_n was solved by using MATLAB (version 2014a, MathWorks, Natick, MA).

Results and discussion

Table 7.1 shows the fermentation results of *Lactobacillus* strains with glucose as carbon source. *L. plantarum* strains consumed almost all of the glucose within 24 h, while *L. delbrueckii* and *L. brevis* consumed glucose more slowly than *L. plantarum* strains; all glucose was consumed within 48 h. Lactic acid yield of *L. plantarum* ATCC 21028 was 0.93 g per g of glucose and Δ*ldhL1*-pLEM415-*xylAB* was 0.96 g per g of glucose, which were very close to the theoretical yield (1 g lactic acid per g of glucose), followed by Δ*ldhL1*-pCU-P*xylAB* , *L. debrueckii*, and Δ*ldhL1*. All glucose was metabolized through EMP in these strains resulting in high yield of lactic acid (Table 7.2). *L. brevis* gave the lowest lactic acid yield due to the diversion of carbon to ethanol and acetic acid production. Small amount of acetic acid was also detected in *L. plantarum* ATCC 21028, Δ*ldhL1*, Δ*ldhL1*-pCU-P*xylAB* and Δ*ldhL1*-pLEM415-*xylAB* strains, which were supposed to ferment glucose through EMP and produce only lactic acid from glucose. The formation of a small amount of acetic acid in these strains could be the semi-anaerobic fermentation condition or glucose limitation, which may cause the conversion of

pyruvate to acetic acid and CO_2 (Kandler 1983). The negative value of f_6 indicated the reverse reaction had occurred (Table 7.2). Acetyl-CoA can be formed from acetate by a reaction catalyzed by acetyl-CoA synthetase, but this reaction is less common than the pyruvate dehydrogenase route; however when acetyl-CoA synthetase route was considered, the calculated carbon flow did not fit into the model.

Table 7.3 shows the fermentation results of using xylose as carbon source. Table 7.4 shows the calculated carbon flow. Lactic acid yield of *L. brevis*, Δ*ldhL1*-pCU-P*xylAB* and Δ*ldhL1*-pLEM415-*xylAB* were 0.50, 0.51 and 0.47 g per g of xylose, respectively, which are close to theoretical yield (0.6 g per g of xylose). Although yield of lactic acid of these three strains were comparable to each other, Δ*ldhL1*-pCU-P*xylAB* and Δ*ldhL1*-pLEM415-*xylAB* consumed more xylose and produced more lactic acid than *L. brevis* within 48 h. With xylose as carbon source, no ethanol was produced in *L. brevis* compared to when glucose was used as the only carbon source. One possible explanation is that NAD^+ must be balanced, and NAD^+ regenerated in lactic acid production compensates the NAD^+ used in xylose fermentation, thus acetyl phosphate can be converted to acetate instead of ethanol to gain one ATP, making PK pathway as efficient as the EMP.

When mixed sugars were used as carbon source, xylose was not used by *L. delbrueckii*, *L. plantarum* ATCC 21028 and Δ*ldhL1* in the mixed glucose and xylose fermentation, and their fermentation performance was not affected by the presence of xylose. The yield of lactic acid of *L. brevis* from mixed xylose and glucose was 0.53 g g^{-1}, lactic acid yield of Δ*ldhL1*-pCU-P*xylAB* was 0.81 g g^{-1} and Δ*ldhL1*-pLEM415-*xylAB* was 0.84 g g^{-1} (Table 7.5). Table 7.6 shows the calculated f_1 to f_{17} value of using mixed sugars. *L. brevis* metabolized both glucose and xylose through PK pathway. Interestingly, about 9 % of consumed glucose was diverted into the PK pathway in Δ*ldhL1*-pCU-P*xylAB* and Δ*ldhL1*-pLEM415-*xylAB* when mixed glucose and xylose were used as carbon source. The reason why glucose was diverted into the PK pathway is still unclear; one possible explanation could be that the PK pathway was used as a bypass in response to lactic acid stress. Pieterse et al. (2005) found out that genes involved in the PK pathway showed an increased expression under lactic acid stress condition. After xylose assimilation plasmid was introduced into *L. plantarum* NCIMB 8826 Δ*ldhL1*, xylose isomerase and xylulose kinase were constitutively expressed and xylose was converted into the common intermediate xylulose-5-phosphate (X5P) which was immediately metabolized in the PK pathway and

converted into lactic acid. Compared to when glucose was used alone, lactic acid production increased to about 38 % for Δ*ldhL1*-pCU-P*xylAB* and 31 % for Δ*ldhL1*-pLEM415-*xylAB*. The increased lactic acid stress may cause the diversion of small amount of glucose into the PK pathway.

Each *Lactobacillus* strain has its own merits or demerits when compared. Selection of the proper strain depends on the final purpose and sugar composition of raw materials. If D-lactic acid is the desired product, and raw materials are rich in lactose, galactose, fructose, or sucrose, such as, cheese whey, sugar cane molasses or juice, then *L. delbrueckii* would be a good choice because it can use all the above mentioned sugars and produce optically pure D-lactic acid through the EMP pathway without any by-product. If high optical purity D-lactic acid is required and the raw material contains mainly glucose, such as glucose enriched stream from lignocellulosic biomass, then Δ*ldhL1* could also be a good choice because Δ*ldhL1* grows faster than *L. delbrueckii* and it can tolerant higher level of oxygen compared to *L. delbrueckii*, which prefers 5 % CO_2 environment during fermentation. If the optical purity of lactic acid is not an important factor, and raw material is rich in glucose, then *L. plantarum* ATCC 21028 can be used. If the raw materials are rich in both glucose and xylose, such as agricultural residues, and D-lactic acid is the product of interest, then either Δ*ldhL1*-pCU-P*xylAB* and Δ*ldhL1*-pLEM415-*xylAB* can be a good option. It seem that *L. brevis* is not a desired strain to produce lactic acid because it metabolizes both glucose and xylose through the PK pathway, and produces significant amount of acetic acid and ethanol, which will enhance downstream processing cost. However, co-cultivation with other glucose fermenting strains, for example *L. plantarum* or Δ*ldhL1*, *L. brevis* will focus on converting xylose into lactic acid and ethanol production will also be reduced (Zhang and Vadlani 2015).

Conclusions

The performances of six *Lactobacillus* strains were compared and the detailed carbon flow was studied for different carbon sources. The existence of different pathways was identified in six different *Lactobacillus* strains. The EMP is dominant in classic homofermentative *L. delbrueckii* strain. The presence of two carbon pathways in facultative heterofermentative *L. plantarum* strains makes the bacteria more flexible with different carbon source. The end product profile of heterofermentative strain *L. brevis* is affected by the oxidation-reduction potential of

the fermentation system. These critical data will help to select proper strain to achieve a desired product with different raw materials; but the performance of these *Lactobacillus* strains on other carbon sources such as lactose, sucrose, fructose, and arabinose needs to be evaluated in detail to provide more information on the strain selection.

Table 7.1 Fermentation results of *Lactobacillus* strain with glucose as carbon source

Strain	Time (h)	Consumed glucose ($g\ L^{-1}$)	Lactic acid ($g\ L^{-1}$)	Acetic acid ($g\ L^{-1}$)	Ethanol ($g\ L^{-1}$)	Yield[1] ($g\ g^{-1}$)
L. delbrueckii	48	30.0±1.9	26.1±1.4	0	0	0.87
L. brevis	48	29.5±1.2	15.0±0.6	6.1±0.1	4.3±0.6	0.50
Lactobacillus plantarum						
ATCC 21028	24	29.6±0.1	27.5±0.2	0.4±0.1	0	0.93
Δ*ldhL1*	24	30.0±0.7	24.6±0.3	0.06±0.1	0	0.82
Δ*ldhL1*-pCU-P*xylAB*	24	29.7±0.7	26.1±0.1	0.6±0.1	0	0.88
Δ*ldhL1*-pLEM415-*xylAB*	24	30.0±0.8	28.7±0.5	0.1±0.2	0	0.96

[1]Yield is calculated by the amount of lactic acid produced divided by the amount of glucose consumed

Table 7.2 Calculation results for carbon metabolic flow with glucose as carbon source (mM carbon)

Strain	f_2	f_3	f_4	f_5	f_6	f_7	f_8	f_9	f_{10}	f_{11}	f_{12}	f_{13}	f_{14}	f_{15}	f_{16}	f_{17}
L. delbrueckii	0	0	0	870	0	870	0	0	0	0	0	870	870	0	0	0
L. brevis	833	500	333	500	130	500	187	203	85	56	28	1000	0	1000	167	833
ATCC 21028	0	0	0	933	-13	913	0	13	20	13	7	933	933	0	0	0
Δ*ldhL1*	0	0	0	823	-2	820	0	2	3	2	1	823	823	0	0	0
Δ*ldhL1*-pCU-P*xylAB*	0	0	0	900	-20	870	0	20	30	20	10	900	900	0	0	0
Δ*ldhL1*-pLEM415-*xylAB*	0	0	0	971	-3	967	0	3	5	3	2	972	972	0	0	0

Table 7.3 Fermentation results of *Lactobacillus* strain with xylose as carbon source

Strain	Time (h)	Consumed xylose ($g\ L^{-1}$)	Lactic acid ($g\ L^{-1}$)	Acetic acid ($g\ L^{-1}$)	Ethanol ($g\ L^{-1}$)	Yield[1] ($g\ g^{-1}$)
L. brevis	48	9.35±0.6	4.7±0.2	4.7±0.1	0	0.50
Lactobacillus plantarum						
Δ*ldhL1*-pCU-P*xylAB*	48	18.8±0.5	9.5±0.6	7.5±0.6	0	0.51
Δ*ldhL1*-pLEM415-*xylAB*	48	17.0±0.6	8.0±0.1	5.9±0.1	0	0.47

[1]Yield is calculated by the amount of lactic acid produced divided by the amount of xylose consumed

Table 7.4 Calculation results for carbon metabolic flow with xylose as carbon source (mM carbon)

Strain	f_1	f_2	f_3	f_4	f_5	f_6	f_7	f_8	f_9	f_{10}	f_{11}	f_{12}
L. brevis	245	245	147	98	245	-58	157	0	157	88	59	29
Δ*ldhL1*-pCU-P*xylAB*	433	433	259	173	433	-76	318	0	250	115	77	38
Δ*ldhL1*-pLEM415-*xylAB*	352	352	211	141	352	-56	267	0	197	85	56	28

Table 7.5 Fermentation results of *Lactobacillus* strain with mixed glucose and xylose as carbon source

Strain	Time (h)	Consumed glucose ($g\ L^{-1}$)	Consumed xylose ($g\ L^{-1}$)	Lactic acid ($g\ L^{-1}$)	Acetic acid ($g\ L^{-1}$)	Ethanol ($g\ L^{-1}$)	Yield[1] ($g\ g^{-1}$)
L. brevis	48	20.7±1.9	2.7±1.9	12.3±0.5	2.6±0.3	5.0±0.1	0.53
Lactobacillus plantarum							
Δ*ldhL1*-pCU-P*xylAB*	48	29.5±0.1	15.0±0.4	36.2±0.5	6.6±0.0	0	0.81
Δ*ldhL1*-pLEM415-*xylAB*	48	29.6±0.1	14.8±0.1	37.5±0.8	7.1±0.1	0	0.84

[1]Yield is calculated by the amount of lactic acid produced divided by the amount of total sugar consumed

Table 7.6 Calculation results for carbon metabolic flow with mixed glucose and xylose as carbon source (mM carbon)

Strain	f_1	f_2	f_3	f_4	f_5	f_6	f_7	f_8	f_9	f_{10}	f_{11}	f_{12}	f_{13}	f_{14}	f_{15}	f_{16}	f_{17}
L. brevis	110	683	410	273	410	186	410	217	87	46	31	15	688	0	688	115	573
Δ*ldhL1*-pCU-P*xylAB*	476	550	330	220	1207	0	1207	0	220	0	0	0	966	877	89	15	74
Δ*ldhL1*-pLEM415-*xylAB*	515	592	355	237	1250	0	1250	0	237	0	0	0	987	895	93	15	77

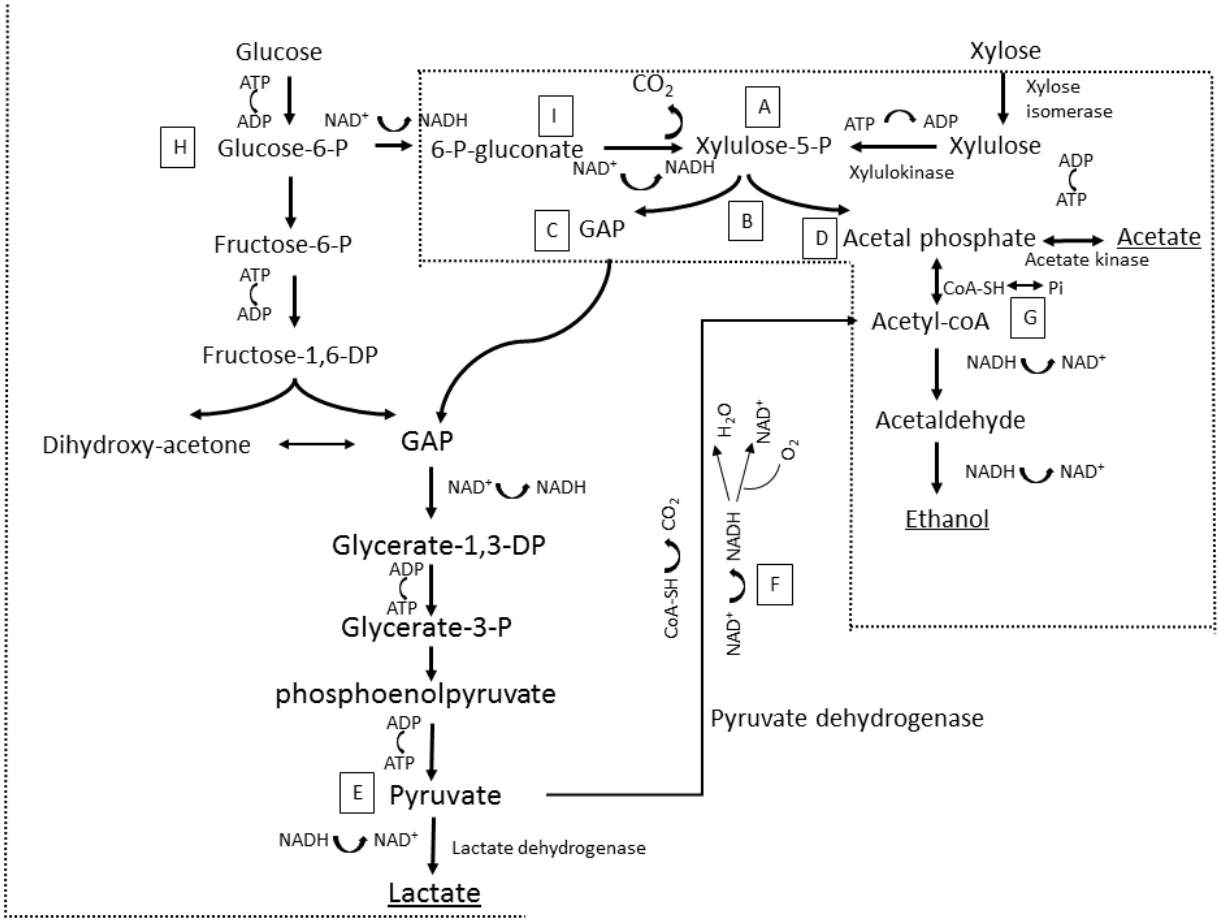


Figure 7.1 Pathway of mixed sugars in *lactobacillus* strains. Junctions A to E corresponded to those in Fig. 7.2, GAP, glyceraldehyde 3-phospahte

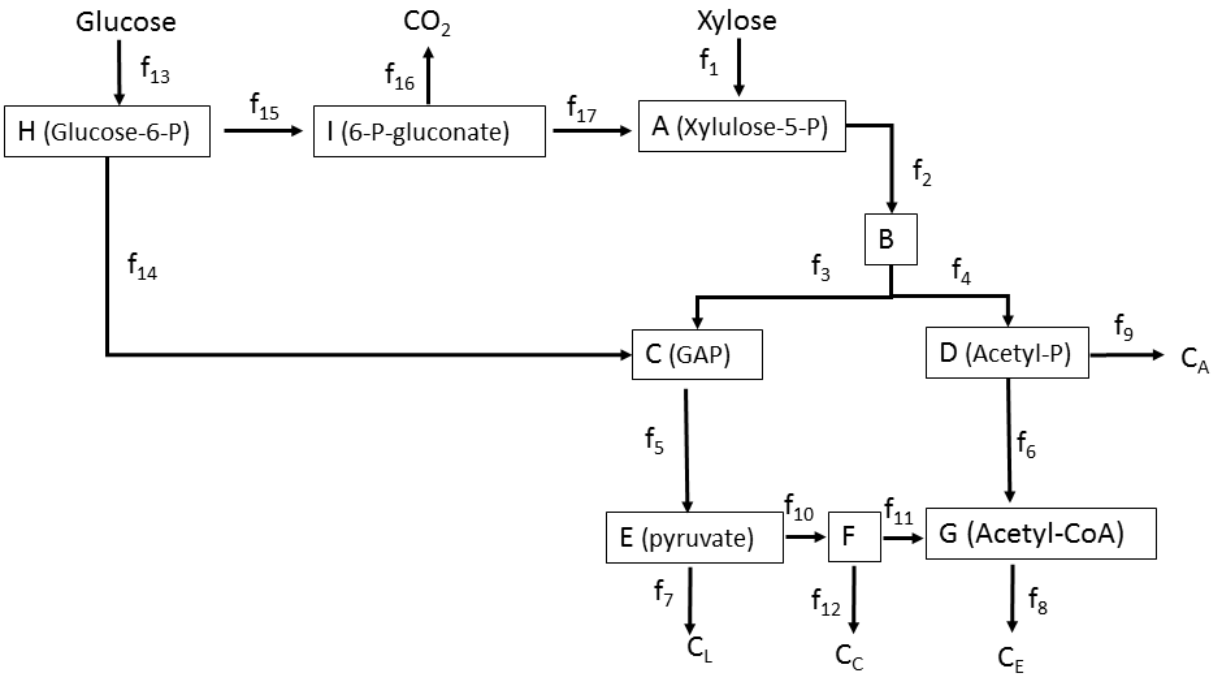


Figure 7.2 Circuit of modified pathways in *Lactobacillus* strains

Chapter 8 - Conclusions and future research

Lactic acid is an industrial important chemical with an expanding market demand because of its multifunctional abilities as a platform chemical. Economics of lactic acid production through fermentation depends on many factors, including the raw materials source, microorganisms, and fomentation process. The purpose of this study was to produce lactic acid, primarily high optical pure D-lactic acid from lignocellulosic biomass using different *Lactobacillus* strains. The following conclusions can be drawn from this study.

1) *L. delbrueckii* produced high level of optically pure D-lactic acid as major end-product from biomass derived glucose, but the dominant sugar xylose derived from hemicellulose was not utilized.
2) L-lactate deficient strain *L. plantarum* NCIMB 8826 Δ*ldhL1* produced optically pure D-lactic acid from glucose and arabinose. A small amount of acetic acid by-product was also produced from the arabinose. Xylose was not used by this mutant strain.
3) *L. brevis* was capable of simultaneously using both glucose and xylose derived from lignocellulosic biomass to produce racemic mixture of D and L-lactic acid. The yield of lactic acid was low compared to *L. delbrueckii* and *L. plantarum* NCIMB 8826 Δ*ldhL1* due to the diversion of carbon to acetic acid and ethanol produciton.
4) Co-fermentation strategy of cultivating *L. brevis* with *L. plantarum* significantly enhanced the yield of lactic acid and minimized the formation of by-products.
5) *L. plantarum* NCIMB 8826 Δ*ldhL1*-pCU-P*xylAB* and *L. plantarum* NCIMB 8826 Δ*ldhL1*-pLEM415-*xylAB* simultaneously used glucose and xylose from lignocellulosic biomass. D-lactic acid was produced at high yield and productivity by these two recombinant strains.
6) SSF process demonstrated the advantages of avoiding substrate inhibition and increasing the productivity and yield of lactic acid compare to SHF process.
7) Soybean meal extract was demonstrated to be a cost-effective substitute to yeast extract for lactic acid production.
8) Metabolic flux analysis based on carbon balance confirmed pathways in different *Lactobacilli* and useful information was attained to make meaningful strain selection for a targeted lactic acid production from a specific lignocellulosic feedstock.

Future research

Currently lactic acid is commercially produced from corn starch or cane sugar via bacterial fermentation. The industrial use of lignocellulosic biomass as feedstocks for lactic acid production by lactic acid bacteria has not been sufficiently profitable due to some limitations as mentioned earlier. Further research in the following area is required to make the lignocellulosic biomass derived lactic acid more economically attractive:

1) *Lactobacilli* strains used in this study need to be evaluated for their tolerance to common inhibitors such as furfural and HMF generated during pretreatment process. *Lactobacilli* growth inhibition was not observed by using alkali pretreated lignocellulosic biomass in this study. However, other pretreatment methods, for example, the acid pretreatment, usually generate much more inhibitors compared to alkali pretreatment; therefore production of lactic acid by *Lactobacilli* using lignocellulosic biomass processed with different pretreatments is required.
2) Lactic acid production from lignocellulosic biomass in this study was performed in laboratory scale, thus scale-up studies are required to further evaluate the scalability and commercial feasibility.
3) High concentration of lactic acid has an inhibitory effect on cellular metabolism and lactic acid production. The end product inhibition caused by lactic acid could result in a disturbance of the regeneration of NAD^+, particularly under anaerobic condition because the NADH oxidase would not be available to convert NADH to NAD^+ in the absence of oxygen. Also the undissociated lactic acid can enter the cytoplasm and disrupt the internal pH and anion pool, which denatures essential enzymes inside the cells. One way to reduce the feedback inhibition of lactic acid is to integrate fermentation with downstream processing. Esterification and hydrolysis is a widely accepted method to purify lactic acid, esterification of lactic acid with ethanol could be an attractive strategy to separate lactic acid from fermentation broth, and promote corn ethanol by adding value to the ethanol production process (Fig. 8.1). The other way is to develop enhanced acid tolerant strain, which can improve lactic acid production and reduce the utilization of neutralizing agents. Acid tolerant strains can be selected by adaptive evolution method. Cells are challenged at low pH with addition of lactic acid for several generations until stable growth is achieved.

4) Although enzyme loading was optimized by RSM in this study, the high cost of enzyme is still a concern for economical production of lactic acid. Cellobiose is the main product in enzymatic hydrolysis of cellulose, and it can strongly inhibit the hydrolysis reaction of cellulase. Therefore, high dosage of β-glucosidase is usually supplemented to reduce the feedback inhibition caused by accumulated cellobiose. By introducing an expression plasmid (Fig. 8.2) carrying genes encoding β-glucosidase into D-lactic acid producing strain, the amount of enzyme used to degrade cellulose will be reduced.
5) Co-utilization of glucose and xylose was achieved by our recombinant strain Δ*ldhL1*-pLEM415-*xylAB*, but it consumes xylose much slower than glucose. The co-utilization of glucose and xylose could be owing to the fact that *clpC* promoter is derived from another lactic acid bacterium, thus the regulation of host strain do not govern its expression or function, and xylose is converted to xylulose-5-P even when glucose is still present. Posthuma et al. (2002) reported that expression of phosphoketolase which catalyzes the reaction of splitting xylulose-5-P into acetyl-P and glyceraldehyde-3-P was induced by pentose sugars and was repressed by carbon catabolite protein A (CcpA) - a key player in carbon catabolite repression (CCR). To improve the xylose uptake rate and productivity of D-lactic acid of this recombinant strain, gene encoding a proton motive force-linked xylose transport can be cloned and co-expressed with xylose assimilation genes (Chaillou et al. 1998) (Fig. 8.3). After the buildup of the xylose transport system in the bacteria, the *ccpA* gene which encodes CcpA could be disrupted and higher phosphoketolase activity could be achieved, which may result in increased conversion rate of xylose into lactic acid.

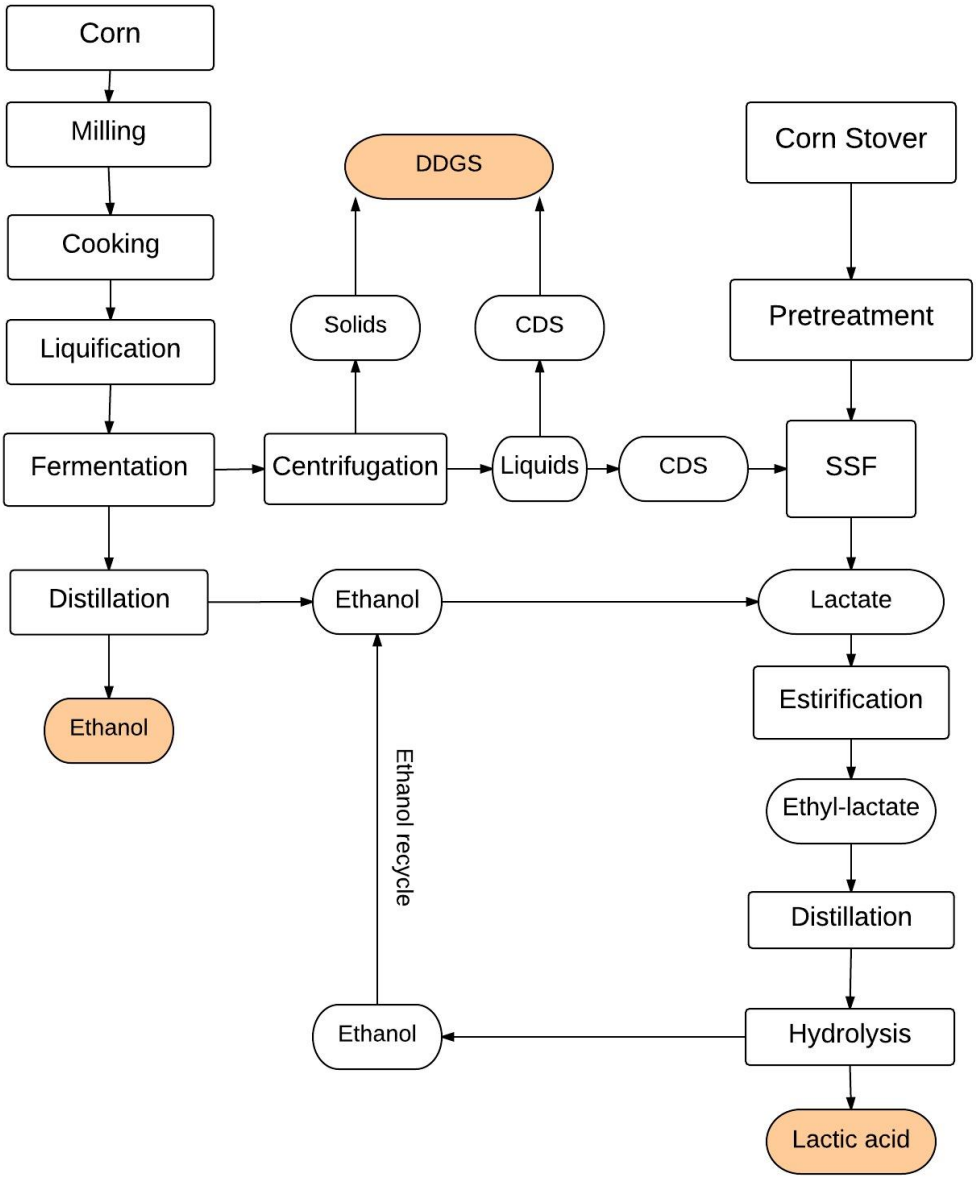


Figure 8.1 Integrated process of lactic acid production with corn ethanol

CDS: Condensed distillers solubles; DDGS: Dried distillers grains with solubles; SSF: simultaneous saccharification and fermentation

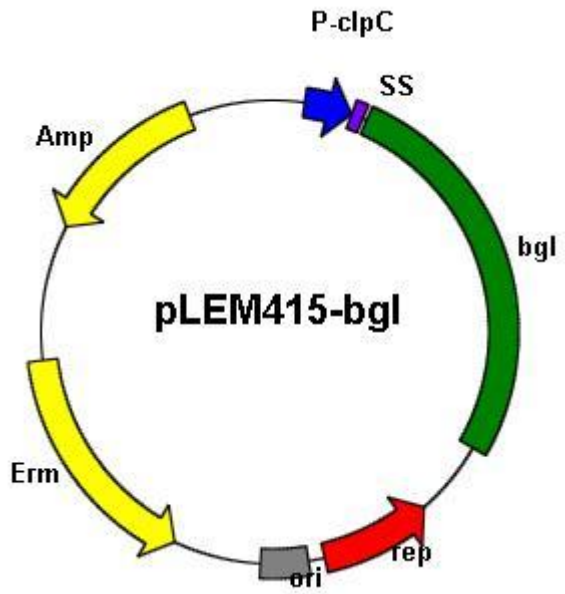


Figure 8.2 β-glucosidase expression vector

bgl: β-glucosidase gene; SS: signal peptide sequence encoding gene; Ori: origin of replication; rep: replication gene from pLEM3; Erm: erythromycin resistance gene from pLEM3; Amp: ampicillin resistance gene from pBII(SK^+); P-clpC: *clpC* promoter from pCU-PxylAB

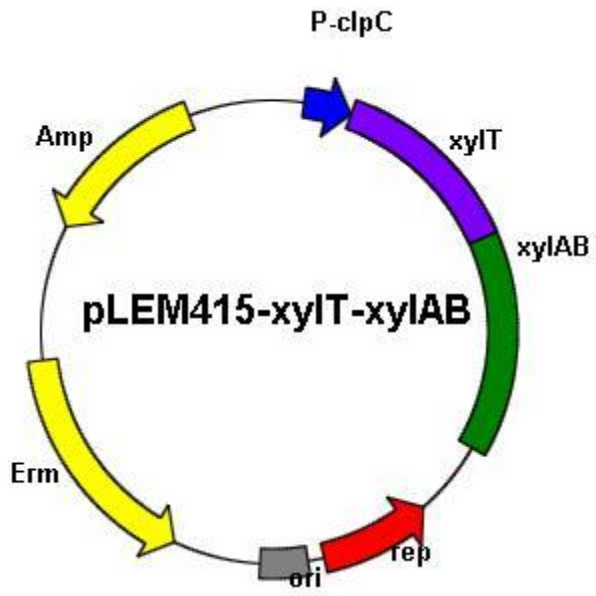


Figure 8.3 xylose symporter and xylose assimilation enzymes co-expression vector

xylT: D-xylose-H^+ symporter genes from *L. brevis*; *xylAB*: xylose assimilation operon from *L. brevis* ATCC 367; Ori: origin of replication; rep: replication gene from pLEM3; Erm: erythromycin resistance gene from pLEM3; Amp: ampicillin resistance gene from pBII(SK^+); P-clpC: *clpC* promoter from pCU-PxylAB

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